Expression of the G_i-coupled RASSL Ro1 in GFAP-positive cells: a novel model of hydrocephalus

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ABSTRACT

ELIZABETH SWEGER: Expression of the Gi-coupled RASSL Ro1 in GFAP-positive cells: a novel model of hydrocephalus
(Under the direction of Ken McCarthy)

Hydrocephalus is a disorder of cerebrospinal fluid dynamics within the central nervous system. Although symptoms of hydrocephalus are often relieved by shunt implantation, complications are common and underlying pathology remains. Understanding the molecular mechanisms of hydrocephalus is critical for development of new therapeutics; however few experimental models allow early events to be studied. Originally designed to study astrocytic G_i signaling, we developed a transgenic mouse line expressing the G_i-coupled RASSL (receptor activated solely by synthetic ligand) Ro1 in GFAP-positive cells by crossing the hGFAP-tTA (tet transactivator behind the human glial fibrillary acidic protein promoter) mouse line with the tetO-Ro1/tetO-LacZ mouse line. Surprisingly, we found that all double-transgenic mice developed hydrocephalus by postnatal day 15, while single-transgenic littermate controls appeared normal.

Hydrocephalic Ro1 mice had enlarged lateral and third ventricles, thinned cortex, partial denudation of the ependymal cell layer, abnormal subcommissural organ, and obliteration of the cerebral aqueduct. Severely affected mice had increased phosphoErk and GFAP expression. Giving breeding pairs doxycycline prevented the expression of Ro1 and onset of hydrocephalus in double-transgenic offspring. Double transgenic mice taken off dox at weaning developed enlarged ventricles within 7 weeks, indicating that Ro1 expression also induces hydrocephalus in adults. Double-transgenic mice injected with a Ro1 inverse agonist

when taken off dox did not develop enlarged ventricles or have ependymal detachment, demonstrating that signaling through Ro1 is required for hydrocephalus. We have discovered a new model that allows onset of hydrocephalus to be controlled, providing the unique ability to study the earliest events in juvenile and adult-onset hydrocephalus.

Ependymal denudation is a common feature of hydrocephalus that appears to occur early in the disease. Positive GFAP and Ro1 staining was detected in a subset of ependymal cells from double transgenic mice off dox. Timed studies showed that Ro1 expression began within three days of dox removal and ependymal denudation began within ten days. Affymetrix gene arrays were utilized to screen for changes in ependymal gene expression at five and nine days after dox removal. No differentially expressed genes were detected between double-transgenic mice and controls at either timepoint; RT-PCR also failed to detect significant changes.

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LIST OF ABBREVIATIONS

ADNF	Activity-dependent neurotrophic factor
BBB	Blood-brain barrier
CNS	Central nervous system
CP	Choroid plexus
CSF	Cerebrospinal fluid
CT	Computed tomography
EC	Ependymal cells
ETV	Endoscopic third ventriculostomy
GFAP	Glial fibrillary acidic protein
GPCR	G protein coupled receptor
HC	Hydrocephalus
ICP	Intracranial pressure
IVH	Intraventricular hemorrhage
KOR	Kappa opioid receptor
LV	Lateral ventricle
MRI	Magnetic resonance imaging
NMDA	N-methyl-D-aspartate
*nor*BNI	*NorBinaltorphimine*
RASSL	Receptor activated solely by synthetic ligand
SAS	Subarachnoid space
TGFβ	Transforming growth factor β

VIP	Vasoactive intestinal peptide

CHAPTER 1
INTRODUCTION

Hydrocephalus. In the United States approximately 1 in 1000 children are born with the neurological disorder hydrocephalus, sometimes commonly referred to as "water on the brain" (Leech and Payne, 1991; Fletcher et al., 1992a; Johanson and Jones, 2001). Children with hydrocephalus (HC) have an imbalance between the production and absorption of cerebrospinal fluid (CSF), resulting in an accumulation of CSF in the ventricles within the brain. If left untreated, the buildup of fluid causes the cranium to expand in an attempt to accommodate the extra fluid and pressure within the brain increases, resulting in disruption and compression of cerebral structures. More than 50% of children with untreated HC die within 10 years, and over 60% of surviving children have neurological impairments. The development of a one-way valve system to drain, or shunt, excess CSF from the brain was developed in the 1950's and was the first breakthrough in HC treatment, greatly improving outcomes for HC patients (Laurence and Coates, 1962; Foltz and Shurtleff, 1963; Becker and Nulsen, 1968). While there have been advances in shunt technology, complications associated with shunt dependence and shunt failure frequently require multiple revision surgeries, making shunting a less than ideal treatment. To date there is no cure for HC, nor is there an effective pharmacological treatment for HC. The discovery of new therapies for HC is complicated by the mulifactoral etiology of HC, and the molecular mechanisms leading the

onset of HC remain poorly understood. Future advances in HC treatment will be greatly aided by experimental models in which the earliest events of the disease can be studied.

We developed a transgenic mouse line expressing the G_i-coupled RASSL (Receptor Activated Solely by Synthetic Ligand) Ro1 in GFAP-positive cells with the aim of studying the role of GPCR signaling in astrocyte-neuronal communication. Expression of Ro1 was restricted to glia by crossing the transgenic hGFAP-tTA (tet transactivator behind the human glial fibrillary acidic protein promoter) mouse line with the transgenic tetO-Ro1/tetO-LacZ mouse line. This cross produced double transgenic mice that expressed Ro1 only in glia. Surprisingly, we found that all transgenic mice expressing Ro1 reliably developed hydrocephalus. We analyzed these mice in an effort to develop a new model of hydrocephalus that will further our understanding of the pathophysiology of the disease. An overview of the ventricular system, cerebrospinal fluid formation and function, and a review of the literature on HC will aid in understanding the Ro1 model.

Anatomy of the ventricular system. The central nervous system (CNS) is bathed and perfused in extracellular fluids that have critical roles in maintaining optimal conditions for brain activity. There are three fluid compartments in the CNS: the ventricles, the subarachnoid space, and the interstitial space. The ventricles and subarachnoid space are filled with cerebrospinal fluid (CSF). The fluid filling the interstitial space is very similar to CSF and is simply called interstitial fluid.

The ventricular system is composed of the lateral ventricles (ventricles one and two), the third ventricle and the fourth ventricle (Figure 1). The ventricles of adult humans hold approximately 30 mLs of CSF (Brodbelt and Stoodley, 2007). The choroid plexi, specialized

tissue responsible for most nascent CSF production, are found in each of the ventricles. The lateral ventricles, the largest of the ventricles, have five distinct regions: the anterior (frontal) horn, body, posterior (occipital) horn, temporal (inferior) horn and the collateral trigone or atrium. The lateral ventricles (LV) are separated from each other by the septum and are each

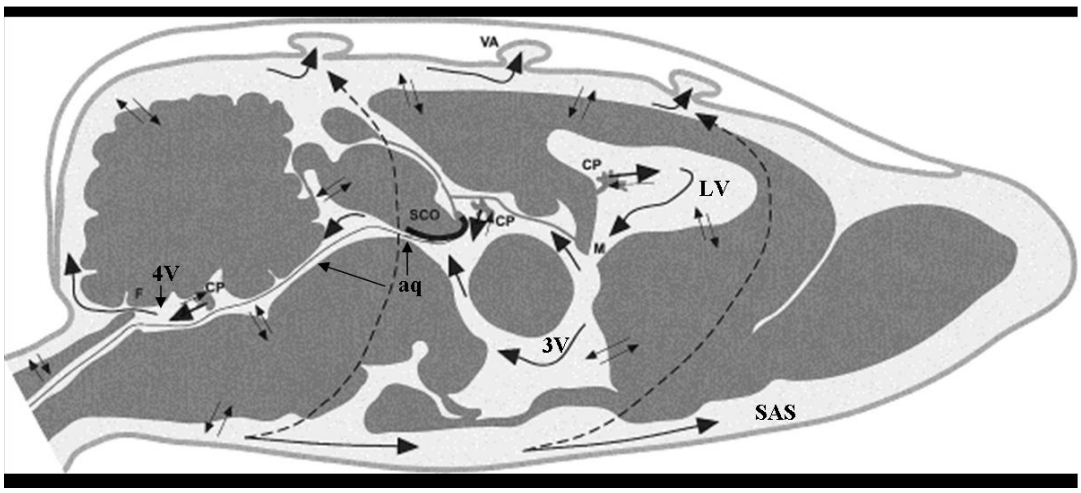

Figure 1. The ventricular system. Cerebrospinal fluid (CSF) is produced in the choroid plexus and flows from the lateral ventricles into the third ventricle through the foramen of Monro, around the thalamus, through the aqueduct of Sylvius into the fourth ventricle, through the foramen of Magendie and formina of Luschka (not shown) into the subarachnoid space. The solid curved arrows show the net movement of CSF flow through the ventricles. The dotted lines represent the movement of CSF through the SAS to the arachnoid villi. Abbreviations: CP=choroid plexus, LV=lateral ventricle, M=foramen of Monro, 3V=third ventricle, SCO=subcommissural organ, aq=aqueduct of Sylvius, 4V=fourth ventricle, F=foramen of Magendie, SAS=subarachnoid space, VA=arachnoid villi.

connected to the mid-line third ventricle via the interventricular foramen of Monro. The third ventricle is irregularly shaped and lies between the thalami and above the hypothalamus. Four recesses project from the third ventricle. The optic and infundibular processes are located rostrally below the LV, while the pineal and supra-pineal recesses are located caudally. The third ventricle is connected to the fourth ventricle by the cerebral aqueduct of Sylvius. The aqueduct is the narrowest part of the ventricular system and is thus most susceptible to obstruction in CSF disorders. The fourth ventricle is triangular in shape and is located at the base of the brain ventral to the cerebellum. The median eminence, one

of the circumventricular organs, lies on the floor of the fourth ventricle. There are three foramina in the roof of the fourth ventricle that connect the fourth ventricle to the subarachnoid space. The medial foramen of Magendie is located caudally and opens into the cisterna magna. The two lateral formina of Luschka connect the fourth ventricle to basal cisterns (Johanson, 2003; McLone, 2004; Di Terlizzi and Platt, 2006).

The subarachnoid space surrounds the brain and spinal cord and contains roughly four times more CSF than the ventricular system. The CNS is covered by three membranes collectively called the meninges (Figure 2). The dura mater is adherent to the skull and contains nerves, blood vessels and venous sinuses. The pia mater tightly covers the external surface of the nervous tissue on top of the superficial glial limiting layer, and is sometimes referred to as the pia-glia. The avascular arachnoid membrane lies in between the dura and pia and bridges the sulci, or grooves, on the surface of the brain. The resulting space

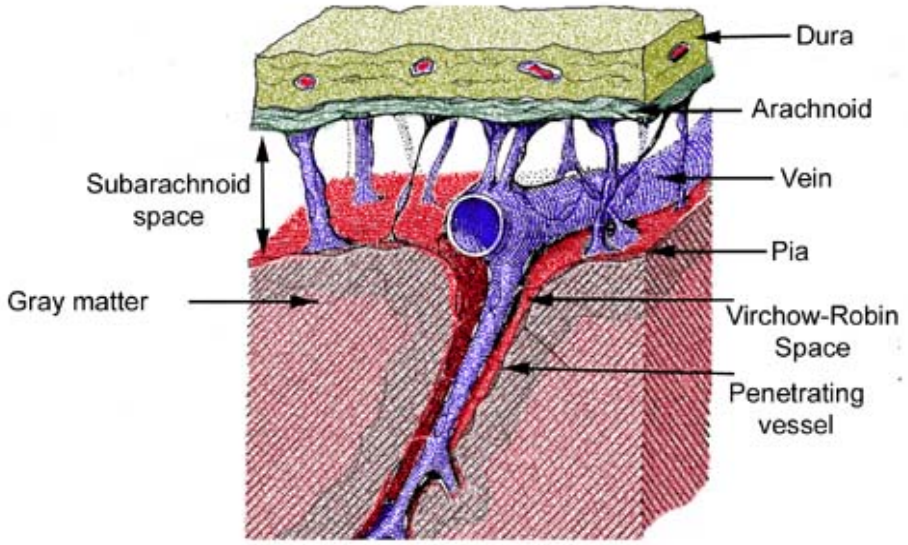

Figure 2. The subarachnoid space. Reprinted with permission www.sbsdefense.com.

between the pia and arachnoid membrane is the subarachnoid space and is filled with CSF. A loose mesh of fibroblasts and collagen, called the arachnoid trabeculae, connects the arachnoid membrane to the pia and may have a role in directing CSF flow (Yasargil et al., 1984; Segal, 1996; Johnston and Teo, 2000; Brodbelt and Stoodley, 2007). Major blood vessels also lie within the subarachnoid space, and are sheathed by a cuff of pia and arachnoid membrane as they penetrate into the brain. CSF also fills these spaces, called Virchow-Robin spaces (Johanson, 2003).

The interstitial fluid immediately surrounds the neurons and glia and is separated from the CSF in the subarachnoid space by the pia and from ventricular CSF by the ependymal layer lining the ventricles. The interstitial space is the largest of the three fluid compartments, holding roughly 280 mL of fluid in the normal human adult. CSF and interstitial fluid are in almost free communication across the interfaces separating them, and thus impact the composition of one another (Johanson, 2003; Brodbelt and Stoodley, 2007).

CSF transports nutrients and other critical substances from the blood to the central nervous system. Because neurons and glia require a precise extracellular fluid composition for optimal functioning, the composition of CSF needs to be tightly regulated. The blood-brain barrier (BBB) and blood-CSF barrier control the exchange of fluid, ions and proteins between the blood and CSF or interstitial fluid. Most blood vessels in the brain are surrounded by endothelial cells tightly joined by zonula occludens (tight junctions), forming the BBB that severely restrict most solutes from diffusing out of the blood. However, in several specialized areas, including the choroid plexus and median eminence, the blood vessel endothelial layers lack tight junctions, allowing a variety of substances to diffuse from the blood into the interstitial space. In these areas the epithelial layer (choroid plexus) or the

ependymal layer (median eminence) has tight junctions on their apical surface, forming a blood-CSF barrier to protect the brain while allowing these organs to carry out their specialized functions. The arachnoid membrane also has tight junctions to prevent CSF from leaking freely into the dural sinuses (Miller and Leslie, 1994; Di Terlizzi and Platt, 2006; Brodbelt and Stoodley, 2007).

The ependymal cell layer that lines the ventricles separates CSF from interstitial fluid, also acting as a CSF-brain barrier. Although ependymal cells (EC) are joined by zonula adherens and gap junctions, allowing CSF and interstitial fluid to readily communicate across the ependyma, the EC layer has features that allow it to serve as a protective barrier. Ependyma are equipped to remove and/or prevent toxic substances from crossing into the nervous tissue (Del Bigio, 1995; Strazielle and Ghersi-Egea, 2000). Metallothionein in EC takes up copper, zinc and heavy metals from the CSF while transferrin binds up iron (Blaauwgeers et al., 1993; Moos and Mollgard, 1993; Suzuki et al., 1994). Removal of copper and iron may protect the brain from oxidative damage since they are involved in ROS (reactive oxygen species) formation (Halliwell, 1992). Ependymal cells have been shown to sequester proteins, tracers, dyes and even latex beads via pinocytosis (Brightman, 1965; Booz and Wiesen, 1976; Broadwell and Sofroniew, 1993), proving they can take up various potentially harmful substances from CSF. Pinocytotic vesicles then fuse to lysosomes where enzymes are available for degradation, preventing re-entry into the brain. Enzymes for degradation of neuropeptides, including aminopeptidase N, dipeptidyl-peptidase, enkephalinase, endo-oligopeptidase, have been localized to EC (Matsas et al., 1986; Bourne et al., 1989; Birch et al., 1990; Oliveira et al., 1990; Schwartz et al., 1991; Gee et al., 1993). Receptors for some drug classes and enzymes for drug metabolism have also been found in

EC (Benavides et al., 1983; Abramovitz et al., 1988), implicating EC in removal of xenobiotics.

There is some evidence that ependyma can function in immune responses as well. Following injections of interferon-γ, ependyma express tissue necrosis factor alpha and major histocompatibility complex II (Tarlow et al., 1993; Lampson et al., 1994). Macrophages are found normally on the apical surface of EC. During infection ependyma have increased expression of vascular adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1) that may further promote macrophage adhesion (Deckert-Schluter et al., 1994; Strazielle and Ghersi-Egea, 2000).

The ependymal layer is capable of regulating interstitial fluid composition. Microvilli on the apical surface of EC have a glycocalyx coating, and sialic acid, poly-N-lactosamine and D-galactose residues could be involved in the transfer of information about CSF chemical composition (Paulson, 1989; Del Bigio, 1995). Receptors for various growth factors are also present, and EC produce both basic and acidic fibroblast growth factor. Since EC do not proliferate *in situ* in response to growth factors, they may regulate some other function besides growth in EC (Del Bigio, 1995). Peptidases involved in the synthesis or activation of CSF-born neuropeptides, such as peptidyl glycine alpha amidating monooxygenase, gamma glutamyl transferase, tissue kallikrein, furin, and angiotensin converting enzyme (ACE), are found in EC (Del Bigio, 1995). ACE is part of the angiotensinogen (possibly supplied from astrocytes) to angiotensin II conversion pathway, and in rats angiotensin II receptors have been localized to EC (Gehlert et al., 1991). Since angiotensin II is involved in regulating peripheral water balance, it may also play in similar role in the CNS. Vasopressin, found in CSF, is known to increase EC permeability to water

(Rosenberg et al., 1986; Sorensen, 1986; Chen et al., 1993). Furthermore, aquaporin channel 4 has been localized to the ependymal basolateral membrane and suggests that movement of water between interstitial fluid and CSF may be regulated by ependyma (Jung et al., 1994). Thus the BBB and brain-CSF barriers regulate the exchange of substances between the blood and the brain and monitor CSF composition, activities critical for the normal functioning of the central nervous system.

Functions of cerebrospinal fluid. CSF also has specific functions critical for maintaining a healthy central nervous system (CNS). First, CSF cushions and supports the brain through a buoyancy effect. CSF is more than 99% water and has a specific gravity nearly equal to that of nervous tissue. As a result, the brain is actually suspended in CSF, reducing the effective weight of the brain by more than 95% and protecting the brain from shearing and tearing forces caused by acceleration and deceleration associated with normal physical activity (Bergsneider, 2001; Johanson, 2003; Brodbelt and Stoodley, 2007). CSF also protects the brain by maintaining a constant intracranial pressure (ICP). As stated by the Monro-Kellie doctrine, blood, brain and CSF are enclosed in a rigid space. Since tissue and fluid cannot compress, a change in the volume of one component must be compensated for by a nearly equal and opposite change in another. As arterial and central venous pressure fluctuates with respiration, exertion and posture changes, CSF volume inversely increases or decreases to maintain ICP within normal ranges. CSF volume is regulated by absorption rates; absorption into venous blood is rapidly enhanced when ICP increases, and is slowed when ICP drops. CSF pressure can change as much as 1-2 mm H_2O with arterial pulse and 2-10 mm H_2O with breathing, illustrating the importance of CSF compensation. In the event of a sudden

increase in intracranial blood volume, CSF can also be temporarily stored in the subarachnoid space (Weller et al., 1992; Segal, 1996; Johanson, 2003; Di Terlizzi and Platt, 2006).

In addition to physically protecting the brain, CSF has many roles related to its circulation through the CNS. Micronutrients are transported by the choroid plexus into CSF for distribution to target cells. Vitamin C, folates, deoxyribonucleosides, vitamin B_6, and trace elements are examples of nutrients that enter brain parenchyma via CSF bulk flow and diffusion across ependymal and pial membranes. CSF also carries hormones and signaling molecules needed for homeostasis, trophic support and neurodevelopment. Growth factors and peptides like transthyretin, insulin-like growth factor, thyroxine, transforming growth factor β (TGFβ), growth hormone, bone morphogenic proteins, arginine vasopressin and fibroblast growth hormone are all transported from the blood to the brain in CSF. CSF circulation is an important part of neuroendocrine signaling as centrally released peptides are carried in CSF to their targeted areas. For example, hormone-releasing factors from the hypothalamus (i.e., gonadotropin releasing-hormone, thyrotropin releasing-hormone and growth hormone releasing-hormone) are carried in the CSF to the median eminence (Johanson, 2003; Di Terlizzi and Platt, 2006; Johanson et al., 2008). CSF helps to regulate hypothalamic and respiratory function by carrying growth factors and peptides to the hypothalamus, medulla oblongata and the choroid plexus (Brodbelt and Stoodley, 2007). Tanycytes, specialized ependymal cells located in the third ventricle of adults, have long processes that extend into the neurophil and wrap around blood vessels. They are believed to be a part of the neuroendocrine system since they form a direct link between CSF and the capillary plexus in the hypothalamus (Di Terlizzi and Platt, 2006; Brodbelt and Stoodley,

2007). Additionally, CNS provides a route for parasynaptic neurotransmitter transmission. Neurotransmitters that leak from synapses are carried in the interstitial fluid along perivascular and subependymal pathways (the ependymal basement membrane is continuous with perivascular membranes) to CSF where they may provide a background signaling "tone" (Johanson, 2003).

Finally, CSF transports metabolic wastes, toxins and ions to sites for removal. Proteins from the breakdown of tissues, macromolecules, cellular debris, ions, xenobiotics, and organic anions such as homovanillic acid and 5-OH-indolic acetic acid (metabolites of dopamine and serotonin, respectively) are carried by CSF to the arachnoid villi where they are absorbed into venous blood (Bergsneider, 2001), to the lymphatic system where they are drained into lymph, or to the choroid plexus where they are removed by transporters. The choroid plexus expresses transporters for a variety of substances, including P-glycoprotein and multidrug-resistant protein for taking up organic anions. These transporters also efficiently clear some drug classes and antibiotics. By draining into lymphatics CSF connects the CNS, which lacks its own lymphatic system, to the peripheral lymphatic system and thus provides the potential for foreign substances in the CNS to trigger an immune response. Furthermore, CSF provides a "sink" for diluting excessive ions that flow into CSF from brain interstices. Potassium and H^+ are two important examples; a buildup of K^+ in interstitial fluid causes seizures while too much H^+ leads to ischemia (Johanson, 2003; Di Terlizzi and Platt, 2006; Brodbelt and Stoodley, 2007).

Clearly CSF plays a critical role in normal CNS function beyond physical protection. CSF is ideally situated to regulate the composition of the interstitial fluid that bathes neurons and glia and to facilitate long-range communication between different brain regions. By

supplying needed nutrients and removing excess waste, CSF helps to maintain interstitial fluid concentrations within the narrow ranges required for optimal neuronal function.

Cerebrospinal fluid production. Choroid plexus tissue has long been recognized as the site of CSF production. There are four choroid plexi in the brain, one in each of the ventricles. Choroid plexi form frond-like projections from the roof of the third and fourth ventricles and from the walls of each lateral ventricle. The choroid plexi in the lateral ventricles are flat and thin, resembling a leaf, while the choroid plexus of the fourth ventricle is more complex and lobular in appearance. The third ventricle choroid plexus is the smallest, and its appearance is a combination of the other two (Strazielle and Ghersi-Egea, 2000). Studies using isolated ventricles and choroid plexus preparations estimate that 60 to 75 percent of CSF formation is from choroid plexus (Johnston and Teo, 2000; Johanson, 2003; Brodbelt and Stoodley, 2007).

Choroid plexus tissue is composed of a single layer of tightly packed cuboidal epithelial cells surrounding a central core containing a capillary bed and loose connective tissue. The choroid plexus epithelial layer is continuous with the ependymal cell layer that lines the ventricles, but unlike the ependyma, the choroid plexus epithelial layer has tight junctions in between the cells on their apical surface. These tight junctions prevent the majority of hydrophilic ions and molecules from crossing the cell layer into the CSF, although the electrical resistance of choroid plexus epithelium is less than that of the BBB (Johanson, 2003). The choroid plexus epithelium folds into many villi around each capillary, creating processes that project into the ventricles. The basolateral membrane of choroid plexus epithelium also has many interdigitations and infoldings. The villi, along with a brush

border of microvilli on the apical surface, greatly increase the surface area of the choroid plexi. Choroid plexi have a surface area of approximately 100 cm^2 per gram tissue, highlighting its importance as a secreting and reabsorbing tissue. Not surprisingly, choroid plexi have a high density of mitochondria and an extensive Golgi apparatus to support its secretory function (Strazielle and Ghersi-Egea, 2000; Johanson, 2003). In addition to CSF production, the choroid plexus acts as a filtration system, removing metabolic waste, foreign substances, and excess neurotransmitters from the CSF. Peptidases in the microvilli of the apical surface regulate peptides in CSF (Brodbelt and Stoodley, 2007). In this way the choroid plexus has a very important role in helping to maintain the delicate extracellular environment required by the brain to function optimally.

While choroid plexi are believed to produce the majority of CSF, they are not the sole source of CSF formation. Removal of the choroid plexus did not abolish CSF formation in isolated ventricle experiments (Hassin, 1948). In 1969, Milhorat removed the choroid plexus (choroid pexectomy) from rhesus monkeys and compared the development of induced hydrocephalus in the plexectomised monkeys to monkeys with intact choroid plexus, and found only small changes in hydrocephalus severity between the two groups (Milhorat, 1969). Further research showed that bilateral choroid plexectomy reduced CSF formation by only 33-40% (Milhorat et al., 1971; Milhorat, 1974). A human case study found CSF production to be within normal range in a 5 year old child who had undergone choroid plexectomy as an infant (Milhorat et al., 1976).

Sources for extraventricular CSF production have not been definitively established; however proposed sources include capillaries and metabolic processes in the parenchyma, ependymal cells, and spinal subarachnoid space (Johanson et al., 2008). The endothelium

surrounding capillaries in the brain appear to be capable of secreting fluid and osmolites. Like choroid plexus epithelium, capillary endothelium is polarized with different ion transporters and channels expressed on the basal and apical surfaces. Na^+K^-ATPase, a pump essential in CSF formation in choroid plexus, is expressed on the apical surface and thus could drive the net secretion of sodium and chloride, the main components of CSF (Segal, 1996; Abbott, 2004). Metabolically, water is produced in cells as a byproduct when glucose is oxidized to CO_2 and could thus contribute to interstitial fluid formation, although not enough water is generated this way to account for the total volume of interstitial fluid (Rapoport, 1976; Abbott, 2004). Ependymal cells have several characteristics suggesting they have secretory capabilities. In rat ependyma, mRNA for the secretory proteins chromaogranin B, secretogranin, and sulfated glycoprotein-2 have been found (Senut et al., 1992; Danik et al., 1993; Gee et al., 1993). Granulophysin, a membrane protein associated with exocytosis, is present in human ependymal cells (Hatskelzon et al., 1993) and surface blebs and vesicles have been observed in the ependymal cells lining the cerebral aqueduct in rabbits and pigeons (Meller and Dennis, 1993; Mestres and Rascher, 1994). During development, secretory droplets can be found on the apical surface of third ventricle ependyma (Booz, 1975). Although there are some reports of CSF from spinal sources (Sato et al., 1973), multiple studies have failed to find significant spinal subarachnoid space CSF production in cats, dogs and monkeys (Lux and Fenstermacher, 1975; del Pozo-Reyes et al., 1978; Hamer and Sahar, 1978), making the subarachnoid space an unlikely source for CSF formation.

CSF is an aqueous solution composed mainly of sodium, chloride and bicarbonate, similar to blood plasma from which it is derived. Nascent CSF contains the following ion

concentrations (in mEq/kg H_20): 158 Na^+, 138 Cl^-, 25 HCO_3^-, 3.3 K^+, 1.7 Ca^{2+}, and 1.5 Mg^{2+}. CSF is not, however, simply a filtrate of plasma. CSF differs from plasma with slightly higher levels of Cl^- and Mg^{2+} and lower levels of Ca^{2+} and K^+ (Johanson, 2003; Di Terlizzi and Platt, 2006). Ca^{2+} and K^+ levels in particular must be kept stable in CSF as small changes in their concentrations impact CNS excitability. Accordingly, changes in plasma concentrations of these ions have little or no effect on CSF concentrations (Crone and Christensen, 1981). Protein, urea, and glucose are present in CSF but at levels 30-40% less than plasma, and amino acids are present in only trace amounts (Johanson, 2003). Normally CSF is clear and colorless and has a total nuclear cell count of only 0-2 cells/µL (Meinkoth et al., 1998); any cloudiness or color is a sign of pathology.

CSF is formed in a two step process, ultrafiltration of plasma from blood and active net secretion of Na^+, Cl^- and bicarbonate ions (HCO_3^-) into the ventricles. Capillaries in choroid plexus are large and have thin, fenestrated endothelial walls, allowing plasma, macromolecules and ions to filter from the blood into choroid plexus interstitial fluid. Under normal conditions, blood flow to the choroid plexus capillaries is quite high, flowing ten times faster than cerebral blood flow (Johanson, 2003), and filtration of plasma is proportional to the hydrostatic pressure gradient between blood and choroid plexus interstitial fluid (Strazielle and Ghersi-Egea, 2000; Johanson et al., 2008).

The net flow of ions from plasma across choroid plexus basolateral and apical membranes and into the ventricular space is driven by the coordinated activity of ion transporters and channels (Figure 3). On the apical membrane, sodium is transported into CSF by ATP-dependent Na^+K^-ATPase and $Na^+K^+2Cl^-$ cotransporters. The activity of these pumps and cotransporters keeps the internal concentration of sodium around 20-30 mM,

establishing a favorable sodium gradient for secondary active transport of sodium across the basolateral membrane by Na^+-H^+ exchange and Na^+-HCO_3^- cotransporters (Bergsneider, 2001; Praetorius et al., 2004; Bouzinova et al., 2005). Along with the bicarbonate ions brought into the choroid plexus (against its concentration gradient) by Na^+-HCO_3^- cotransporters, bicarbonate is accumulated in the cell as carbonic anhydrase hydrates CO_2 into HCO_3^- and H^+. The buildup of HCO_3^- allows chloride ions to be brought into the choroid plexus epithelium by Cl^--HCO_3 exchange in the basolateral membrane. Chloride may also enter by a K+ - Cl- cotransporter as the KCC3 isoform has been localized to the

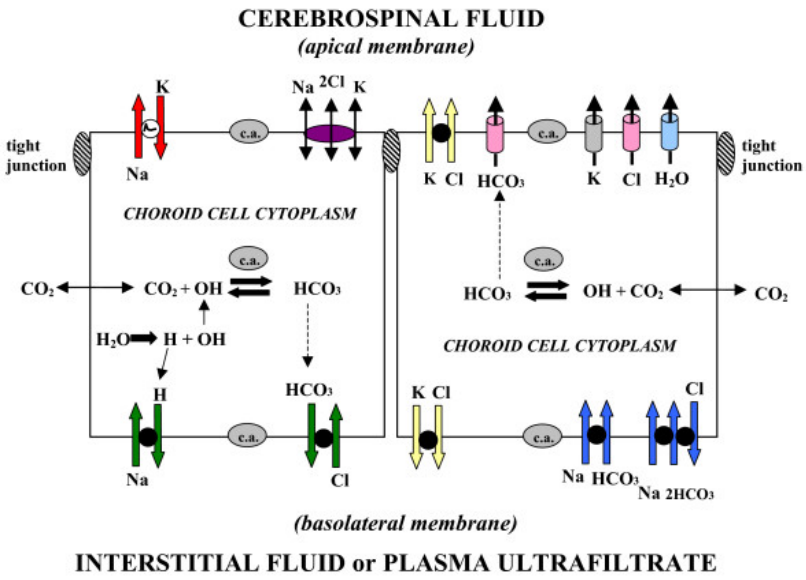

Figure 3. Choroid plexus epithelium and the production of cerebrospinal fluid. CSF production, the net flow of bicarbonate (HCO_3), chloride (Cl^-) and sodium ions (Na^+), occurs through the coordination of channels, transporters and cotransporters in the choroid plexus epithelium. HCO_3^- accumulates in the cytoplasm via Na^+- HCO_3^- cotransporters and Cl^-- HCO_3^- exchange pumps in the basolateral membrane and by carbonic anhydrase (c.a.) activity. HCO_3^- and Cl^- enter the CSF through inward rectifying anion channels in the apical membrane. Sodium is actively pumped into CSF by apical membrane Na^+K^+ ATPase and Na^+K^+ $2Cl^-$ cotransporters, creating a favorable concentration gradient that allows sodium to enter the cell via Na^+-H^+ exhange and Na^+-HCO3$^-$ cotransporters in the basolateral membrane. Abbreviations: Na=sodium ion, K=potassium ion, HCO_3=bicarbonate ion, c.a.=carbonic anhydrase, Cl=chloride ion. Reproduced in agreement with the BioMed Central open access policy. Johanson *et al. Cerebrospinal Fluid Research* 2008 **5**:10 doi:10.1186/1743-8454-5-10

basolateral membrane (Pearson et al., 2001). Inward rectifying anion channels in the apical membrane allow chloride and HCO_3^- to enter CSF by moving down their respective electrochemical gradients. The resulting hyperosmolar solution causes water to move into CSF via aquaporin 1 channels on the choroid plexus apical membrane (Bergsneider, 2001; Brown et al., 2004; Johanson et al., 2008). There is, of course, reverse movement of ions due to reabsorptive fluxes to the blood, and other ions such as calcium and magnesium are moved between blood and CSF; however these fluxes, although important for CNS function, are not instrumental in CSF production (Johanson, 2003; Brown et al., 2004).

CSF is made continuously and is estimated to turn over three times in twenty-four hours in humans, which corresponds roughly to 600 mL of new CSF/day in healthy adults (Johanson, 2003). Choroid plexus (human) produces CSF at 0.3-0.6 mL/min/g, although there is some question as to whether CSF is produced constantly at this rate. In a quantitative MR study monitoring CSF flow, CSF formation followed circadian rhythms, with a maximum rate of production at 2:00 AM and a minimum rate of production at 6:00 PM (Nilsson et al., 1992). There are other factors that influence CSF production as well. The choroid plexus receives neural input via sympathetic imput; sympathetic stimulation decreases CSF production while removal of sympathetic imput greatly increases production. Although the choroid plexus receives cholinergic and peptidergic innervations, their influences on choroid plexus function are not well understood (Johnston and Teo, 2000).

The role of ICP and blood flow on CSF production also remains unclear. As mentioned previously, the rate of CSF production is dependent on the hydrostatic pressure gradient between choroid plexus interstitial fluid and blood plasma. Thus it seems likely that changes in blood flow and ICP would affect the rate of CSF production. Blood flow to the

choroid plexus is regulated by endocrine and neurohumoral imputs (Shapira et al., 1992; Strazielle and Ghersi-Egea, 2000; Johanson et al., 2008) that seem ideally suited for controlling CSF formation. Yet experiments manipulating interstitial fluid and blood flow have had conflicting results on CSF formation. For example, administration of acetazolamide, an inhibitor of carbonic anhydrase, caused a 50% decrease in CSF formation while simultaneously increasing choroidal blood flow (Faraci et al., 1990). A similar result is seen with vasoactive intestinal polypeptide (VIP). When given intraventricularly, blood flow increased 20% yet CSF formation dropped by 30%. Choroidal blood flow also increased when VIP was administered i.v., but CSF formation remained unchanged (Nilsson et al., 1991). Conversely in acute hydrocephalus, increased ventricular CSF pressure causes an increase in choroid plexus interstitial fluid pressure, reducing plasma filtration and subsequent CSF formation. Blood flow to the choroid plexus is also reduced in HC (Johanson et al., 2008). Overall, most evidence suggests that within the rather narrow limits of normal physiology, CSF production is relatively independent of both ICP and blood flow (Bergsneider, 2001). It is conceivable that the observed decreases in CSF formation are compensating for increased ICP caused by increased blood flow in CNS, however this area remains controversial.

Cerebrospinal fluid absorption. The classic pathway of CSF movement in the CNS (Figure 4) is for CSF to circulate by bulk flow from its site of origin at the choroid plexus, through the ventricles and basal cisterns, down the posterior side of the spinal cord, up the anterior side, and through the cranial subarachnoid space to the midline convexity where it is absorbed into the dural sinuses by arachnoid villi (Welch and Friedman, 1960; Arkhinova,

1966; Di Chiro, 1966; Du Boulay, 1966). CSF movement is believed to be driven by new CSF production, postural effects, pulse waves generated by the choroid plexus, arterial or respiratory pulsations, and cilia movement (Gonzalez Santander et al., 1984; Roth et al., 1985; Johnston and Teo, 2000; Bergsneider, 2001). The arachnoid villi (also known as pacchionian granulations or arachnoid granulations) are invaginations of the arachnoid membrane into the dural sinuses. Their role in CSF absorption was first described by Welch and Friedman (1960), who believed they contained one-way valves that open when CSF pressure exceeds venous pressure. Radiocisternograms, where large molecule radioisotope-labeled tracers are injected into CSF, showed an accumulation of tracers over the cerebral convexity where the arachnoid villi are located (Di Chiro, 1966; Bergsneider, 2001). Together these studies were interpreted as evidence for the arachnoid villi being the main site of CSF absorption (Milhorat, 1987; Bergsneider, 2001; Di Terlizzi and Platt, 2006).

It is now evident that CSF movement through the CNS is more complex than originally described. Flow-sensitive MR imaging shows bulk flow of CSF through the ventricular system but no net flow around the spinal cord or towards the cerebral convexity (Schossberger and Touya, 1976; Greitz, 1993; Bergsneider, 2001; Abbott, 2004). Instead, CSF movement in the subarachnoid space and around the spinal cord is pulsatile in nature, which is caused by changes in brain blood volume during the cardiac cycle. Blood flows into the brain during systole and out during diastole, causing the brain to move up and down like a piston as subarachnoid CSF is dispelled to accommodate increased blood volume. During systole, CSF in the subarachnoid space and cisterns moves into the compliant spinal canal, while CSF in the brain moves first retrograde through the aqueduct and foramen of Magendie and then moves anteretrograde at mid-systole. During diastole CSF again moves retrograde

through the aqueduct and foramen (Greitz et al., 1997a; Bergsneider, 2001). This pulsatile CSF flow could account for the concentration of tracers around at the arachnoid villi. Furthermore, in radionuclide cisternography studies there is a second site of tracer accumulation located at the lumbar-sacral sac that is unaccounted for by the bulk flow model (Greitz and Hannerz, 1996). As new CSF flows from the foramina of Lusckae and Magendie it mixes with and dilutes the tracers. The accumulations of tracer in radiocisternograms are thus due to a lack of dilution since these sites are the furthest away from the flow of new

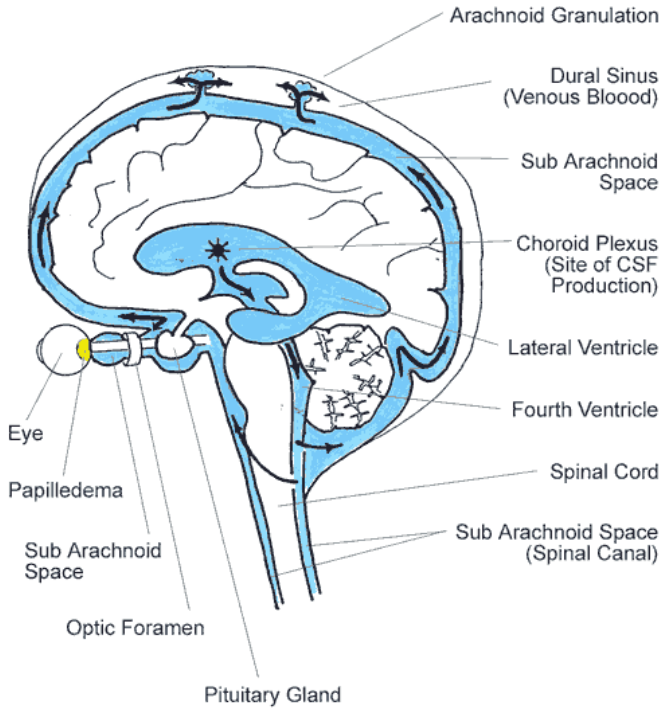

Figure 4. **Classic view of cerebrospinal fluid circulation.** Cerebrospinal fluid is produced by the choroid plexus and moves by bulk flow through the lateral, third, and fourth ventricles into the subarachnoid space. After flowing through the spinal canal and around the brain, CSF is finally absorbed into the dural sinus via the arachnoid granulations (or arachnoid villi). Image obtained from www.corkrey.com

CSF.

The evidence against CSF circulating via bulk flow raises the question of CSF absorption. If most CSF does not move to the arachnoid villi, then where is it absorbed?

Even before the pulsatile movement of subarachnoid CSF was demonstrated, the arachnoid villi as the primary site for CSF drainage were a topic of debate. Human infants do not develop arachnoid villi until the fontanels fuse, meaning there must be other routes for CSF drainage; and in spite of numerous references to valve-like channels in arachnoid villi, no such structures have ever been found (Greitz et al., 1997a, b). Furthermore, other studies found various tracers appeared in blood or nervous tissue faster than could be accounted for by bulk flow. Albumin, inulin and sucrose injected intrathecally into rats reached half-maximal concentrations in the blood and CSF in 100, 80 and 30 minutes, respectively. By bulk flow it should take 6-8 hours to reach the arachnoid villi (Reed and Woodbury, 1963; Greitz et al., 1997a, b). In 1972, iodinated albumin injected into lumbar subarachnoid space was absorbed directly into the spinal canal (Soderborg and Lying-Tunell, 1972). CT citernography showed that contrast media penetrates into brain parenchyma (Drayer and Rosenbaum, 1977), and albumin and x-ray contrast media were found in blood only minutes after injection into the subarachnoid space (Greitz et al., 1997a). When radioactive water was injected into the carotid artery, nearly all was found to penetrate the brain and almost 50% returned to blood within two minutes, indicating that the movement of water and the movement of solutes are not necessarily the same (Oldendorf, 1970; Greitz et al., 1997a; Bergsneider, 2001). This was confirmed by comparing 3H-water and 3H-inulin injected into the lateral ventricles of cats. The 3H-water was rapidly absorbed into adjacent cerebral capillaries while 3H-inulin moved bidirectionally along the ventricular system and was slowly absorbed into blood (Bulat et al., 2008; Vladic et al., 2009).

The current concept of CSF flow and absorption focuses on the nasal lymphatic system as a primary site for CSF drainage. Lymph and CSF have been thought to interact for

over a century, but work within the last decade has shown that the majority of CSF in multiple mammalian species (including human and non-human primates) flows out of the subarachnoid space along the perineural spaces of the olfactory nerves, through the cribriform plate, and into the submucosa, where lymphatic ducts form a collar around the olfactory nerves. CSF is ultimately returned to venous blood through the cervical lymphatics (Abbott et al., 1985; Bradbury, 1985; Johnston et al., 2004; Zakharov et al., 2004; Johnston et al., 2005; Koh et al., 2005; Johanson et al., 2008). Spinal nerve rootlets also connect with lymphatic vessels, and CSF is absorbed similarly, although on a smaller scale, in the spinal cord (Johanson et al., 2008).

A secondary site of CSF absorption is believed to be through blood vessels in the parenchyma. Water moves freely between blood and interstitial fluid, and larger molecules (albumin, iodide, horseradish peroxidase) are evidently able to enter the blood, probably through carriers or by pinocytosis (Greitz et al., 1997a). CSF in the subarachnoid space enters the parenchyma through Virchow-Robin spaces (Figure 2) and across the ependymal layer, preferentially flowing in the subependymal space, white matter tracts, and around blood vessels. Once in the parenchyma, CSF mixes with interstitial fluid and can be absorbed through capillary endothelium (Brightman, 1965; Abbott, 2004).

Although arachnoid villi are no longer believed to be the primary site of CSF absorption, further investigation into their function has suggested they do have a role as an auxiliary drainage route when CSF pressures are elevated (Johnston et al., 2004; Johanson et al., 2008). Significant concentrations of soluble and particulate tracers are found in the dural sinuses when CSF-sinus blood pressure gradients exceed 20 cm H_2O, but not at physiological pressure gradients of 5 cm (Mann et al., 1979; McComb, 1983, 1992; Johnston et al., 2004).

The original thought that CSF moves through the arachnoid villi via valve-like structures is questionable, however. Large vacuoles seen in the basal surface of arachnoid villi epithelium and the presence of pinocytotic vesicles on the endothelium of sinuses indicate that fluid is transported into blood instead of flowing through an open channel (Johnston and Teo, 2000).

The ventricular system and CSF carry out multiple functions within the CNS, providing physical protection, communication routes, and a means for removing harmful substances from the brain. The physiology of CSF production, means of movement through the CNS, and routes of absorption are clearly not fully understood but are obviously critical for normal functioning of the tightly regulated environment of the CNS. A disturbance in any of the components (i.e. CSF production, physical pathways, or absorption routes) has serious consequences, including hydrocephalus. Hydrocephalus can result from perturbations of any and all parts of CSF physiology, and is described further in the next chapter.

CHAPTER 2

HYDROCEPHALUS: CLASSIFICATION, CAUSES, PATHOLOGY, AND TREATMENT

Hydrocephalus (HC) is a multifactoral, progressive brain disorder characterized by accumulation of cerebrospinal fluid (CSF) in the brain and subsequent expansion of the ventricles. HC is the most common neurosurgical problem in children with an incidence of 1-2 cases per 1000 live births (Peacock and Currer, 1984; Leech and Payne, 1991; Casey et al., 1997; Johanson et al., 2001; Fletcher et al., 2002). The advent of better diagnostic tools has allowed adult onset of HC to be recognized as an increasing health issue. Approximately 69,000 to 80,000 new cases of HC (both adult and childhood) are diagnosed each year in the United States (Bondurant and Jimenez, 1995), although the actual number of cases may be underreported since HC in adults can mimic other neurological disorders (Casmiro et al., 1989a).

Classification

Communicating vs. non-communicating**.** HC cases can be divided into two broad categories, communicating vs. non-communicating. Either type may be congenital, where the patient is born with HC, or acquired, where HC develops after birth. Both congenital and acquired HC can be acute, where the ventricles are enlarging and intracranial pressure (ICP) is high and/or unstable, or chronic, where ventricles are enlarged but ICP is normal or slightly elevated but

stable. Frequently the same initial insult can cause multiple types of HC, depending on when the insult occurs in conjunction with undetermined genetic and environmental factors. For example, intracranial hemorrhage leading to HC can occur during gestation, thus causing congenital HC, or at any age after birth, causing acquired HC. The cause of hemorrhage can vary from genetic disorders to traumatic injury, and the resulting HC can be communicating or non-communicating, acute or chronic. Although various systems for classifying HC can be found, the communicating vs. non-communicating system seems to be the most common.

HC in which the ventricular system is free from physical obstruction is called communicating HC. Communicating HC thus must result from either increased production or decreased absorption of CSF, although cases where CSF flow is blocked outside the ventricular system (i.e., in the basal cisterns or parasagittal arachnoid villi) are frequently included in this category. Despite the extremely diverse etiology of HC, abnormalities of CSF circulation leading to HC are usually due to compromised absorption into venous blood; HC due to increased production of CSF is extremely rare (Mori et al., 1995; McAllister and Chovan, 1998; Johanson et al., 2001). Bleeding into the ventricles and infection of the meninges result in compromised CSF absorption and are the leading causes of communicating HC (Beni-Adani et al., 2006). Non-communicating HC occurs when physical obstruction of the ventricular system prevents normal CSF flow. Congenital abnormalities, tumors, genetic defects, stenosis of the aqueduct, meningitis and hemorrhage are common sources of obstruction (Vertinsky and Barnes, 2007).

Chronic hydrocephalus. In 1965 Hakim and Adams described cases of HC where intracranial pressure (ICP) was normal and coined the term “normal pressure

hydrocephalus", or NPH to describe this condition. Today it is recognized that there are several different types of HC without elevated IPC and the term chronic hydrocephalus is used to describe these cases. Chronic HC is defined as all cases with both ventriculomegaly (Evans index greater than 0.3) and normal or chronic low-grade increase of ICP (Edwards et al., 2004). This definition includes compensated (arrested) HC, *ex vacuo* HC, adult onset stenosis of the aqueduct (Harrison et al., 1974; Oi et al., 2000) and normal pressure hydrocephalus (NPH), both secondary NPH where HC occurs after a clear insult (i.e., meningitis, SAH, trauma) and idiopathic NPH where no clear precipitating factor is present (Hebb and Cusimano, 2001; Zemack and Romner, 2002). Chronic HC is seen most frequently in older children or adults. Like acute HC, chronic HC has multiple precipitating causes and has a similar pathology, although chronic HC progresses more slowly as the adult brain appears better able to compensate than the immature or developing brain (Edwards et al., 2004).

The actual number of chronic HC cases and its rate of development is unknown. In the US, adult cases of chronic HC are estimated at approximately 40,000/year, comprising more than half of new hydrocephalus cases (Bondurant and Jimenez, 1995). Other studies estimate 2.6 new cases per 100,000 people each year world-wide (Hoglund et al., 2001). Although not all cases of chronic HC have cognitive impairments, chronic HC can present symptoms that are diagnosed as dementia, and an estimated 5-10% of patients with dementia are thought to actually have chronic HC (Fisher, 1982; Hakim et al., 2001; Kuba et al., 2002; Vale and Miranda, 2002).

Compensated congenital hydrocephalus, also known as arrested HC, occurs when the ventricles spontaneously cease enlarging and stabilize (Whittle et al., 1985). Increased CSF

absorption by circumventricular and intraaxial blood vessels is thought to account for the halt of ventricular expansion, although cases of spontaneous ventriculostomy are not uncommon. Compensation occurs in 10-15% of congenital HC cases, and usually occurs when the child is between 1 and 2 years of age (Schick and Matson, 1961; Hochwald et al., 1969; Nonaka et al., 2008). Compensation does not mean patients are healthy, however. An estimated 2/3 of untreated children with compensated HC have below normal intelligence, and progressive loss of cognitive function may occur throughout adulthood (Laurence and Coates, 1962; Yashon, 1963; Oi et al., 2000; Edwards et al., 2004; Nonaka et al., 2008). They also have an increased risk of epileptic seizures and sudden death (Rickert et al., 2001; Black and Graham, 2002). Although the brain has compensated for the initial cause of HC, there is little tolerance for further insult to CSF pathways, so even modest straining, exertion or injury can cause instantaneous death from ischemia in cardio-respiratory centers in the brainstem (Botez et al., 1977; Dobkin, 1978; Dickerman et al., 2003; Edwards et al., 2004). The deterioration of cognition in adulthood further suggests that HC is not fully compensated and is actually progressing at a very slow rate. Children with compensated HC are generally not shunted, but newer research suggests shunting would be beneficial as neurological damage does occur once a threshold of ventricular enlargement is reached. The slower the expansion, the larger the ventricles can be before significant axonal damage and cognitive and motor impairments occur, which would explain the delay in onset of symptoms (Del Bigio et al., 2003).

Ex vacuo hydrocephalus. *Ex vacuo* hydrocephalus occurs when brain parenchyma atrophies, resulting in enlarged ventricles (Bradley, 2001b). *Ex vacuo* HC historically was not considered to be related to CSF hydrodynamics, but this conclusion is controversial

(Silverberg et al., 2003; Silverberg, 2004). Proper CSF circulation is being recognized as important for progenitor cell proliferation and migration, particularly in the germinal matrix of the subventricular zone, since CSF carries growth factors and other signaling molecules necessary for normal development. Neuronal death, loss of white matter, and defects in neuronal precursor proliferaton and migration can all result in loss of parenchyma (Johanson et al., 2001). While disrupted CSF hydrodynamics is not the only cause of improper development, it is conceivable that *ex vacuo* HC could be related to loss of proper CSF flow.

Causes of hydrocephalus

Physical malformations**.** Developmental malformations that physically change the ventricular system frequently have HC as part of their pathology. The most common developmental cause of HC is Chiari II (or Arnold-Chiari) malformation, where the cerebellum and brainstem extend into the foramen magnum and block normal CSF flow. Approximately one third of infantile HC cases are caused by Chiari II malformations (Vertinsky and Barnes, 2007). A second physical cause of HC is Dandy Walker malformation, a developmental abnormality in which the cerebellum is underdeveloped, resulting in a misshapen fourth ventricle that drains CSF into a cyst. Approximately 1 in 5000 infants is born with Dandy-Walker malformation (Parisi and Dobyns, 2003), and approximately 20-80% of cases develop HC after the neonatal period due to obstruction of the formina of Magendie and Luschka (Vertinsky and Barnes, 2007). Other less common developmental abnormalities also cause HC, but will not be covered here.

Stenosis of the aqueduct**.** The aqueduct of Sylvius is the narrowest part of the ventricular system and closure, or stenosis, of the aqueduct is a frequent finding in both acquired and congenital HC. Primary stenosis is caused by developmental abnormalities such as forking, septation and gliosis. Onset of HC from primary stenosis generally occurs during the neonatal period or in infancy. Secondary stenosis can occur after various types of insult, including hemorrhage, infection and tumors (Vertinsky and Barnes, 2007). Stenosis of the aqueduct also occurs in HC cases without a clear precipitating factor. Approximately 45% of congenital HC cases have no known cause, and fetal HC with aqueduct stenosis constitutes a major portion of these cases (Castaneyra-Perdomo et al., 2006). Whether stenosis is the cause of HC or a consequence of HC in these cases is unknown, and contradictory evidence from experimental models has made this a topic of debate.

Infection. Infection blocks drainage of CSF from the ventricular system by causing inflammation of the meninges, limiting the flow of CSF through the subarachnoid space to absorption sites. Meningitis is, along with hemorrhage, one of the most common causes of congenital HC, but also causes HC in infants, children and adults. Congenital HC from infection occurs when sub-clinical viral infections in pregnant women infect the fetus; post-natal meningitis can be acquired via sinus or ear infections. Sources of infection include gram-negative bacteria, group B streptococcus, pneumococcus, *Listeria*, neisseria, tuberculosis, cytomegalovirus, toxoplasmosis, mumps, rubella and varicella (Margolis and Kilham, 1969; Herndon et al., 1974; McComb, 1997; Vertinsky and Barnes, 2007).

Hemorrhage. Intraventricular hemorrhage (IVH) is a complication of premature birth (Cherian et al., 2004a; Crews et al., 2004) and occurs in 12-20% of preterm births where the infant weighs less than 1500g. Intraventricular hemorrhage in preterm births (under 32 weeks gestation) results from the combination of a very rich blood supply to the subventricular zone (required to support the high metabolic requirements of this region during development), fragile blood vessels due to lack of glial support, occasional hypoxia (due to respiratory complications), and unregulated swings in arterial and venous pressure. Together these events cause bleeding into the ventricles. Red blood cells and their breakdown products can block the ventricular system or arachnoid villi, leading to HC (Cherian et al., 2004a). Post-hemorrhagic ventricular dilation (PHVD) affects 15-35% of infants with IVH. Motor dysfunction is seen in approximately two thirds of PHVD infants while one third have cognitive dysfunction (Davis et al., 1987; Sheth, 1998; Kazan et al., 2005).

Severe traumatic brain injury can also result in hemorrhage and subsequent HC. HC may occur from IVH, subarachnoid hemorrhage (SAH), blood clots that block CSF drainage, or from venous compression caused by swelling. Glial scars that form after injury can also hinder proper CSF flow. While onset of HC can occur within weeks of the insult, in some cases HC develops very slowly and takes months, even years, to become evident.

Genetic disorders. Known genetic abnormalities leading to HC account for a very small percentage of HC cases. X-linked recessive mutations in the neural cell adhesion molecule (CAM) gene for L1 neural cell adhesion molecule are known to cause HC (Van Camp et al., 1993; Weller and Gartner, 2001). There are case reports of inherited HC that presents as an

autosomal recessive disorder in some families, but no genes responsible have been identified (Johanson et al., 2001). However, the large number of experimental HC models resulting from genetic alterations suggests that other cases of human HC with unidentifiable causes may also have a genetic component.

Molecular mechanisms of hydrocephalus

HC has an extremely diverse etiology and is found as part of a variety of neurological disorders. Yet the progression and outcomes of HC are similar, implying that some common underlying molecular mechanisms are present. These underlying molecular mechanisms have yet to be fully elucidated and HC remains poorly understood, due at least in part to the difficulty in differentiating between changes that are a consequence of HC from those that are causing HC. Research from experimental HC models has provided some valuable insights into the pathology of HC which are summarized below.

Disregulation of cytokines. In addition to secreting CSF, the choroid plexus secretes a variety of growth factors necessary for proper CNS function, including FGF-1, FGF-2, TGF-β1, NGF, HGF-SF and IGF-II. Signaling molecules present in CSF are important for regulating cell growth and development. The subventricular zone lies very close to the lateral ventricles and contains progenitor cells that continue to proliferate into adulthood. The proximity of the subventricular zone to CSF suggests that the growth factors present in CSF would impact proliferation in this region. Areas of the brain more distant from the ventricles are also affected by CSF contents. For example, Cajal-Retzius cells, which regulate the laminar organization of the cortex, send fibers from their location in the marginal

zone to the ventricles during development, implying that during development the CSF contains factors important for normal cortical development (Johanson et al., 2001). Accordingly, changes in cortical layering and progenitor proliferation have been observed in HC.

Restriction of CSF flow in HC likely causes signaling molecules to become concentrated in the ventricles and alterations in cytokine levels could account for observed changes in progenitor cells. In support of this theory, altered cytokine levels are found in CSF from various experimental models of HC, and CSF obtained from affected H-Tx rats inhibits the proliferation of cultured neuronal precursor cellsfrom normal controls *(*Vetsika et al*.*, 1999*;* Owen-Lynch et al*.*, 2003*).* If the concentration of growth factors in the CSF is changed in HC, one would expect a compensatory feedback on their production. Research investigating the choroid plexus *in vitro* showed a decrease of EGF, FGF-2, and TGF- β1 mRNA and a 10-fold increase in IGF-II mRNA in the choroid epithelium from HC animals (Johanson et al., 2001).

While the observed changes in CSF may very well be a consequence of HC, altered levels of TGF-β1 and FGF-2 levels have been shown to cause HC (Galbreath et al., 1995; Wyss-Coray et al., 1995; Pearce et al., 1996). Ventricular infusions of recombinant basic fibroblast growth factor (FGF-2) in rats and transforming growth factor β1 (TGF-β1) in mice both result in chronic HC characterized by ventriculomegaly, reduced CSF formation and increased fibrosis and collagen deposits in arachnoid villi with a patent aqueduct and normal ICP. In rats given FGF-2, enlargement of the lateral ventricles begins after 2-5 days of infusions and ependymal cells over proliferate, forming rosettes that protrude into the third ventricle (Tada et al., 1994; Johanson et al., 1999b). Two separate transgenic models of HC

were developed by over-expressing TGF-β1 (Kitazawa and Tada, 1994; Tada et al., 1994; Galbreath et al., 1995; Wyss-Coray et al., 1995). In both models TGF- β1 is expressed under control of the GFAP promoter and results in communicating HC. The time of onset and severity of HC in both models correspond to the level of TGF- β1 expression (Crews et al., 2004); in one model, low expressing mice that are clinically normal develop hydrocephalus following injury (Wyss-Coray et al., 1995). TFG- β1 is also elevated in a rat model of post-hemorrhage ventricular dilation (Cherian et al., 2004b). In human HC cases, increased risk of developing chronic communicating HC is associated with elevated levels of CSF TGF- β 1 in patients with subarachnoid hemorrhage (SAH) (Flood et al., 2001) and increased TGF- β 1 expression is found in preterm births with post-hemorrhagic hydrocephalus (Kitazawa and Tada, 1994; Whitelaw et al., 1999).

The TGF- β family of growth factors regulates development, cell growth, differentiation, homeostasis, tissue repair, and deposition of extracellular matrix proteins (ECM) (Roberts and Sporn, 1996; Benveniste, 1998). All three isoforms of TGF-β and their receptors are expressed in neurons, microglia, and astrocytes (Flanders et al., 1991; Wang et al., 1995; Bottner et al., 1996; Ata et al., 1997; Slotkin et al., 1997; Lippa et al., 1998). Overexpression of TGF-β1 in perivascular astrocytes results in increased deposition of ECM proteins like collagen, laminin and fibronectin. This may interfere with the ability of CSF to be absorbed into the vasculature (Cohen et al., 1999). TGF-β1 tg mice also have decreased expression and reduced activity of matrix metalloproteinase 9 (MMP9) along with an altered expression pattern of its inhibitor, tissue inhibitor of MMPs 1 (TIMP1). MMP9 breaks down ECM proteins, and decreased MMP9 activity could contribute to the accumulation of fibronectin and laminin found in the ECM surrounding the microvasculature, the choroid

plexus and the arachnoid granulations (Del Bigio, 1993; Wyss-Coray et al., 1995; Zechel et al., 2002). Together these findings suggest that CSF absorption and flow of interstitial fluid could be impacted by the thickened ECM and thus lead to the development of spontaneous hydrocephalus. Observed abnormal migration of cortical neurons during development may also contribute to the pathology (Crews et al., 2004).

Ependymal cell denudation. Loss of ependymal cells lining the lateral ventricles and the aqueduct is commonly seen in human cases of HC as well as in animal models (Page and Leure-duPree, 1983; Jimenez et al., 2001; Dominguez-Pinos et al., 2005). Although ependymal denudation is considered by some to result from stretching of the ventricles as CSF accumulates (Sarnat, 1995; Kiefer et al., 1998), there is a significant amount of research indicating that the loss or damage of ependymal cells can in and of itself cause hydrocephalus. Loss or damage of ependyma may lead to HC because regulation of bulk flow of fluids between the brain parenchyma and CSF is lost (Rodriguez, 1976; Sarnat, 1995; Jimenez et al., 2001; Batiz et al., 2006). A study of eight human fetuses with communicating HC showed progressive ependymal cell loss as early as 16 weeks gestation when HC was only moderate, making loss of ependyma due to stretching unlikely (Johnson and Johnson, 1968; Margolis and Kilham, 1969; Nielsen and Baringer, 1972; Nielsen and Gauger, 1974; Bruni et al., 1988b; Jones and Bucknall, 1988; Yamada et al., 1991; Dominguez-Pinos et al., 2005). In one large scale study of a spontaneous HC model, Rodriguez et al (2006) found that ependymal denudation and abnormal neurogenesis (stemming from molecular and cellular level abnormalities) preceded the onset of HC in a set temporal and spatial pattern,

and strongly states that the changes seen in the brain (ie, loss of ependyma, closure of the aqueduct) are not the result of mechanical processes but are causal in the pathology of HC.

Although multiple experimental HC models have ependymal denudation, denudation in the *hyh* mutant mouse model of HC has been the most extensively studied. *Hyh* mice are a spontaneous HC model recently discovered to have a point mutation in the gene encoding the alpha-SNAP protein. Although how a decrease in alpha-SNAP causes HC is not understood, a programmed denudation of ependymal cells begins on E12 before the mice develop enlarged ventricles and continues until P14. The pattern of denudation follows the pattern of ependymal maturation, thus in HC mice the areas where ependyma mature sooner (ie lateral ventricles) also have denudation sooner (Jimenez et al., 2001; Wagner et al., 2003; Batiz et al., 2006).

There are two distinct phases in the development of HC in the hyh mouse. During embryonic development the lateral ventricles, the fourth ventricle, and the floor of the aqueduct undergo denudation and the rostral end of the aqueduct becomes stenosed. This is followed by the onset of moderate HC. After birth, the caudal and dorsal walls of the aqueduct undergo denudation, followed by the obliteration of the caudal region of the aqueduct. This obliteration occurs within the first postnatal week, preferentially during the first 3 days (56%). This is also the time when in normal mice the ependymal cells of the aqueduct lose vimentin expression and undergo ciliogenesis (Wagner et al., 2003). Following obliteration of the aqueduct, the mice develop severe HC.

Denudation follows the developmental maturation of ependyma (rostral to caudal, ventral to dorsal), and developmental defects in brain structures correlates with areas of denudation. Not all ependymal cells detach, however. Patches of specialized ependyma that

resist denudation are found on the circumventricular organs, the dorsal and ventral walls of the rostral end of the aqueduct, in a well defined region of the floor of the aqueduct, and on the wall of the collicular recess. This suggests that a defect in the neuroepithelium/ependymal cell lineage may be responsible for both abnormal development of brain structures and hydrocephalus (Paez et al., 2007).

Scanning electron microscopy of the ventricle walls shortly after birth revealed numerous macrophages in areas that had recently undergone denudation. Detached ependyma are frequently associated with clusters of macrophages. Detaching ependymal cells appear normal, ie, not dying, and have a smooth, spherical appearance (Jimenez et al., 2001; Wagner et al., 2003). Macrophages also are found on areas that will soon undergo denudation and contact exclusively multi-ciliated ependyma. Macrophages are notably lacking on areas that had undergone denudation early in development and on the specialized ependyma that never detach.

Wagner et al (2003) postulated that ependyma in *hyh* mice may have a defect in glycosylation of *N*-linked sailoglycoproteins, resulting in galactose as the terminal sugar residue. This defect would only occur at the stage of differentiation when that enzyme would be active, which would explain the link between differentiation and detachment. Macrophages have receptors for galactose, which could explain the interaction of macrophages with ependymal cells prior to denudation. Ependymal denudation and ependymal rosettes in the neuropil are seen in chick embryos after N-cadherin is blocked by a single injection of antibody into the CSF (Ganzler-Odenthal and Redies, 1998), demonstrating that interfering with surface proteins can lead to ependymal denudation and abnormal migration. Ependymal denudation and sub-ependymal rosette formation are also

seen in infants with post hemorrhage ventricular dilation and in a rat model of post hemorrhage ventricular dilation, although changes in N-cadherin or other surface proteins has yet to be demonstrated (Fukumizu et al., 1995, 1996; Takano et al., 1996; Cherian et al., 2004a).

Subcommissural organ abnormalities. Improper functioning of the SCO is another abnormality associated with HC. Although the exact link is not established, an abnormal SCO has been described in multiple congenital and experimental HC models and in humans (Overholser et al., 1954; Takeuchi et al., 1987; Bruni et al., 1988b; Irigoin et al., 1990; Yamada et al., 1992; Castaneyra-Perdomo et al., 1994; Danielian and McMahon, 1996; Louvi and Wassef, 2000; Estivill-Torrus et al., 2001; Jimenez et al., 2001; Perez-Figares et al., 2001; Blackshear et al., 2003; Baas et al., 2006; Sweger et al., 2007). The SCO is postulated to regulate electrolytes and monitor pressure (Dominguez et al., 1993) and is known to secrete the negatively charged glycoproteins that form the Reissner fiber. The Reissner fiber (RF) extends the length of the aqueduct and may function to prevent the stenosis or obliteration of the aqueduct (Overholser et al., 1954) by repelling the negatively charged glycocalyx of the ependyma. In support of this theory, the sialic acid residues of the RF face the aqueductal lumen (Fernandez-Llebrez et al., 1987; Wagner et al., 2003) and the main RF glycoprotein SCO-spondin contains multiple anti-adhesive microdomains (Gobron et al., 1996; Monnerie et al., 1998; Meiniel, 2001). Loss of the RF is sufficient to cause aqueductal stenosis and HC (Cifuentes et al., 1994; Perez-Figares et al., 1998; Vio et al., 2000; Wagner et al., 2003). Since the SCO is a specialized type of ependyma, an ependymal cell lineage defect could also be responsible for SCO abnormalities.

The SCO also secretes soluble glycoproteins and is, along with the choroid plexus, the first secretory organ to develop in the brain (Somera and Jones, 2004). SCO glycoproteins would be able to diffuse throughout the ventricles during development, prior to the directional CSF flow that develops after communication between the ventricular system and the subarachnoid space is established (Jones and Sellars, 1982; Somera and Jones, 2004). In support of this theory, the choroid plexus has receptors for SCO-spondin, an important SCO glycoprotein that is also implicated in neuronal maturation (Gobron et al., 1996; Gobron et al., 2000; Meiniel, 2001). Soluble SCO sialoglycoproteins may be important for maintaining intact ependymal cell layers, since loss of ependymal surface sialic acid residues is sufficient to cause detachment, leading to obliteration of the aqueduct and HC (Grondona et al., 1996).

Altered levels of SCO secretions and glycoproteins have been found in CSF from animal models of HC and in human fetuses with HC (Irigoin et al., 1990; Rodriguez et al., 1990; Bonadio et al., 1992; Castaneyra-Perdomo et al., 1994; Castaneyra-Perdomo et al., 2006; Martinez-Pena y Valenzuela et al., 2006; Gonzalez-Marrero et al., 2007). Castaneyra-Perdomo et al (2006) found an additional four RF glycoprotein (AFRU) bands in multiple types of human HC that were not present in normal CSF, while Martinez-Pena y Valenzuela et al (2006) found 5 additional bands in the CSF of spontaneously hypertensive rats with ventricular dilation. SCO AFRU immunoreactivity is also reduced in these rats. In WKY rats, several protein bands (AFRU) are absent from the CSF of both spontaneous and induced HC, and the SCO immunoreactivity for AFRU is also reduced (Gonzalez-Marrero et al., 2007).

Changes in SCO glycoprotein immunoreactivity precede the onset of HC in several experimental models. LEW/Jms rats have a poorly developed SCO and posterior commissure and an abnormally shaped dorsal midbrain. These abnormalities precede closure of the aqueduct and onset of HC (Yamada et al., 1992). In H-Tx rats, reduction in RF glycoprotein immunoreactivity (AFRU) in the SCO is seen at E16, while the closure of the aqueduct and the loss or reduction of SCO ependymal cells does not begin until E17 and the onset of HC does not occur until P0. Loss of glycoprotein staining was always found in HC rats, while closure of the aqueduct and ventricular dilation were not always present (some HC rats had dilated ventricles but open aqueducts, while some had closed or narrowed aqueducts but normal ventricles. However, most had both enlarged ventricles and narrowed or closed aqueduct). Embryos with low AFRU reactivity also showed delayed maturation of the brain prior to E17 (Somera and Jones, 2004). These findings suggest that some pathological process involving the SCO is in place prior to the aqueduct narrowing or closing, and this process is exacerbated or accelerated when the aqueduct closes.

Loss of cilia function. Choroid plexus epithelial cells and the ependymal cells lining the ventricles and circumventricular organs are a mixture of single and multi-ciliated cells. Functions of cilia can vary, depending on their type. Motile cilia are involved in the transport of fluids (Satir and Sleigh, 1990) and intracellular signal transduction. A large protein complex, the IFT particle, moves along the cilia axoneme via kinesin II complex (anterograde) and dynein motor proteins (retrograde). This movement is called intraflagellar transport (IFT) and is important in intracellular signal transduction (Kozminski et al., 1993; Rivkin et al., 1997; Pan et al., 2003; Wang and Snell, 2003). Immotile cilia are less

understood, but are believed to regulate ion transport and act as mechanosensors, similar to immotile cilia in the kidney where they detect flow of fluid through the collecting ducts (Lee et al., 1999; Choi et al., 2001; Praetorius and Spring, 2001; Banizs et al., 2005). Immotile cilia also have a possible role in chemoreception. Neuronal primary cilia have olfactory receptors, somatostatin-3 (SST3) receptors and serotonin receptors (Handel et al., 1999; Brailov et al., 2000). All of these functions could have roles in regulating the formation and composition of CSF.

Hydrocephalus frequently is a component of the complex pathologies associated with stunted or absent cilia in animal models (Bruni et al., 1988a; Torikata et al., 1991; Chen et al., 1998; Taulman et al., 2001; Kobayashi et al., 2002; Sapiro et al., 2002; Zhang et al., 2005) and is also seen in human cases of primary ciliary dyskinesia (PCD) (Jabourian et al., 1986; De Santi et al., 1990; Picco et al., 1993; Zammarchi et al., 1993; al-Shroof et al., 2001; Wessels et al., 2003). HC develops in mice lacking regulatory factor X (RFX3), a transcription factor that regulates ciliogenesis. The SCO is also abnormal in these mice, with reduced SCO-spondin expression and undifferentiated ependymal cells (Baas et al., 2006). Mutations in Polaris (IFT88, *Tg737*^{orpk}), wimple (IFT172), Spage6, Hfh-4 (a transcription factor), polymerase λ, and kinesin II (Kif3A and Kif3B) complexes all result in cilia dysfunction and HC in mutant mice (Marszalek et al., 1999; Murcia et al., 2000; Nonaka et al., 2008). It remains unclear whether cilia abnormalities result from HC or are a contributing cause (Taulman et al., 2001), although in mice lacking the ependymal-specific axonemal dynein heavy chain *Mdnah5,* loss of ependymal flow (the movement of CSF through the aqueduct) due to dysfunctional motile cilia precedes stenosis of the aqueduct and the postnatal development of HC (Ibanez-Tallon et al., 2004).

In *Tg737*^{orpk} mutants, ventricle ependyma have significantly fewer cilia and the cilia that are present are shortened and abnormal (Taulman et al., 2001). However, motile cilia dysfunction is not believed to be the cause of HC in *Tg737*^{orpk}, as the pathology develops on P1, prior to motile cilia development (which occurs around P3). Rather, the immotile cilia on choroid plexus epithelial cells likely have a sensory role, similar to primary cilia in renal tubules. Accordingly, CSF from *Tg737*^{orpk} mice has a high chloride concentration, indicating a dysfunction in ion transport. An inwardly rectifying chloride channel on the apical surface of choroid plexus epithelial cells is responsible for transporting chloride into CSF. This channel is regulated by cAMP, and intracellular cAMP levels are elevated in choroid plexus epithelium (Banizs et al., 2005). Isolated mutant choroid plexus has lower internal pH and increased Na^+-dependent HCO_3^- transport activity, resulting in increased CSF production. Inhibiting PKA activity reverses the phenotype to normal; conversely, normal choroid plexus can be converted to mutant phenotype by adding dibutyryl-cAMP (Banizs et al., 2007).

Clearly, the role of cilia dysfunction in HC pathology is far from determined. Interestingly, proteins essential for cilia formation and function are also required for normal cell cycle progression in non-ciliated cells (Mahjoub et al., 2002; Kim et al., 2004; Bahe et al., 2005; Yang et al., 2006). It is tempting to speculate that changes in cell populations observed in HC models could be linked to some of these proteins, but additional research is needed to understand the connection between cilia function and the pathology of HC.

Oxidative stress. Oxidative stress is increasingly recognized as a contributing factor of various neurological disorders and could be responsible for part of the pathology in HC. NO, a free radical, is toxic at elevated concentrations and nNOS (neuronal nitric oxide synthase)

is strongly upregulated in striatum and periventricular regions in affected H-Tx rats (P21) (Socci et al., 1999). It remains unclear if increases in nNOS expression are causative or compensatory. The NOS/NO/cGMP signaling pathway has been shown to inhibit the Na-K-ATPase activity in choroid plexus, implying that CSF production could be changed by increased NO production. Little research has been done to date on the role of reactive oxygen species in HC, so oxidative stress presents an area for further investigation (Johanson et al., 2001; Johanson and Jones, 2001).

Aging. Changes associated with normal aging in the brain may contribute to the development of adult-onset HC. Turnover of CSF declines with age, allowing potentially toxic agents, such as amyloid beta, to accumulate within the brain. This accumulation is enhanced in patients with hydrocephalus and could contribute to cognitive decline. Reduced CSF production associated with aging is caused at least in part by thickening of the choroid plexus basement membrane, reduced perfusion of the choroid plexus and increased vasopressin levels (Serot et al., 1997a; Serot et al., 1997b; Johanson et al., 1999a; Czosnyka et al., 2001; Preston, 2001; Serot et al., 2003). Absorption of CSF also declines with age as the leptomeninges (leptomeningeal fibrosis) and arachnoid villi become thickened, and deposits of amyloid beta in these tissues may further impair absorption of CSF (Bellur et al., 1980; Bech et al., 1997; Hamano et al., 1997; Albeck et al., 1998). Regression of the subcommissural organ is found in human adults, although the functional significance of this finding is not known (Rodriguez et al., 2001). There is also a reduction in the production and circulation of anti-oxidants and free radical scavengers in the CSF, including glutathione peroxidase and vitamins C and E (Cloez et al., 1989; Tayarani et al., 1989; Czosnyka et al.,

2001; Preston, 2001; Silverberg et al., 2002; Serot et al., 2003; Edwards et al., 2004). All of these changes are typical of the aging brain, and thus do not cause HC in and of themselves. However, there could be underlying factors that make a subset of individuals susceptible to HC, and preventing or reversing age-related changes could be therapeutic in these cases.

Hypertension. Reabsorption of CSF through parenchymal routes (i.e., absorption into venous blood) is now believed to be a significant component of CSF circulation. Hypertension reduces the ability of blood vessels to absorb CSF, causing CSF to accumulate within the brain (Haidri and Modi, 1977; Shukla et al., 1980; Casmiro et al., 1989b; Newton et al., 1989; Bradley et al., 1991; Krauss et al., 1996; Boon et al., 1999; Tullberg et al., 2001). Spontaneously hypertensive rats have progressive ventricular dilation accompanied by reduced subcommissural organ AFRU (glycoprotein) immunoreactivity and altered CSF protein composition (Ritter et al., 1988; Martinez-Pena y Valenzuela et al., 2006). Again, although not every patient with hypertension develops HC, hypertension increases the risk of developing HC. Elevated intracranial pressure can also result from hypertension and cerebrovascular disease. Veins are thought to account for 70-80% of the capacitance in the brain, adjusting blood flow to compensate for increases in intracranial pressure. Hypertension and closure of veins due to cerebrovascular disease thus reduces cerebral compliance. This is of particular concern for patients with compensated HC since cerebrovascular compromise increases the risk of decompensation (Bergsneider et al., 1998; Bateman, 2000; Bradley, 2001a; Czosnyka et al., 2001; Hakim et al., 2001; Yau et al., 2002; Cirovic et al., 2003; Edwards et al., 2004).

Genetic mutations. Mutations in the gene for L1 (L1CAM) are the only genetic mutations known to cause HC in humans. L1 is a neural cell adhesion molecule involved in axon guidance, neuronal migration, signal transduction pathways, and is implicated in learning and memory (Brummendorf and Rathjen, 1995; Scholey et al., 1995; Hortsch, 1996; Fransen et al., 1997; De Angelis et al., 2002). There are at least 70 different mutations of L1 in humans, all of which result in corpus callosum hypoplasia, mental retardation, adducted thumbs, spastic paraplegia and hydrocephalus (CRASH syndrome) (Bickers and Adams, 1949; Fransen et al., 1995; Wong et al., 1995; Kenwrick et al., 1996; Fransen et al., 1997). Other human cases of inherited HC have been documented, but the responsible genes remain unidentified. There are numerous genetic mutations in experimental animals that have HC as part of the resulting phenotype; these may lead to the identification of other genetic HC susceptibilities in humans. There are too many models to cover in detail here, however several well-known and relevant mutations are summarized below.

The suppressor of cytokine signaling (SOCS) family of proteins is involved in the termination of cytokine and growth factor signaling (Starr et al., 1997; Krebs and Hilton, 2000). Approximately 50% of SOCS7 null mice develop HC and die by 15 weeks of age. The SCO is disorganized and is the only apparent abnormality, although the aqueduct was not specifically mentioned as being open and normally shaped (Krebs et al., 2004). Although its specific function is not known, SOCS7 is expressed primarily in the brain and binds to insulin receptor substrate 2 (IRS-2), IRS-4, p85 subunit of PI3K (phosphatidylinositol-3 kinase), vinexin, Nck, Ash, phospholipase Cγ and epidermal growth factor receptors (Matuoka et al., 1997; Martens et al., 2004). SOCS7 may regulate other tyrosine kinase pathways, including Erk1/2, Akt, c-Kit (Mooney et al., 2001; Bayle et al., 2004). The

appearance of HC in SOCS7 null mice thus supports the evidence for disregulation of growth factors in the mechanisms of HC.

Pituitary adenylate cyclase-activating polypeptide (PACAP) receptor type 1 (PAC1) is a G protein coupled receptor for PACAP that is expressed throughout the CNS. Both PAC1 null mice and transgenic mice overexpressing PAC1 develop HC (Hannibal et al., 2001; Otto et al., 2004; Lang et al., 2006). In transgenic mice overexpressing PAC1 the severity of HC correlates with the number of transgene copies. Neuronal proliferation in the cortex and subcommissural organ is decreased at E15.5. Other findings include increased apoptosis, reduced corpus callosum, and thinning of the cerebral cortex, particularly layer V.

PAC1 activates both PKA and PKC, and mice lacking the PKC substrates MARCKS (myristoylated, alanin-rich C-kinase substrate), MARCKS-related protein, p190 RhoGAP or the PKA substrate CREB (cAMP response element-binding protein) also have enlarged ventricles, reduced corpus callosum and other neural tube defects, highlighting the importance of these signaling pathways (Chen et al., 1996; Rudolph et al., 1998; Brouns et al., 2000; Huang et al., 2002). Significantly, two of the susceptibility loci for HC the H-Tx rat include the PAC1 and PACAP genes (Jones et al., 2001a; Jones et al., 2001b; Lang et al., 2006). Ependymal cells express PAC1 and tg PAC1 mice have fewer cilia that are shorter and disorganized compared to normal controls. Phospho-CREB is elevated in tg cilia, suggesting disruption of PKA/PKC signaling could be responsible (Lang et al., 2006).

Hydrocephaly with hop gait (*hyh* mouse) is a spontaneous autosomal recessive mutant born with severely dilated ventricles and a small cerebral cortex. Affected mice have a programmed denudation of the ependymal cell layer in the lateral ventricles and aqueduct that corresponds with specific time points in ependymal differentiation (Jimenez et al., 2001;

Wagner et al., 2003). *Hyh* mice have a missense mutation M105I in Napa, the gene for soluble N-ethylmaleimide sensitive factor attachment protein alpha (α-SNAP). The mutation results in a 40% reduction of α-SNAP protein levels in homozygous mice. Alpha-SNAP is important for intercompartmental transport of proteins, apical protein localization and cell fate determination; *hyh* mice have abnormal localization of proteins like beta-catenin and E-cadherins (Chae et al., 2004; Hong et al., 2004). This mutation apparently affects ependymal cell lineage, although ependymal detachment may be the result of improper localization of adhesion molecules.

Hydrocephalus3 (hy3) mice also have a spontaneous autosomal-recessive mutation that causes lethal HC. The *hy3* mouse mutant is caused by rearrangement in the *Hydin* gene in which exons are rearranged or deleted, causing a premature stop codon. This mutation causes defective CSF reabsorption (Lawson and Raimondi, 1973; Davy and Robinson, 2003). More recently two transgenic mouse lines, hy3 and OVE459, were generated in which the inserted transgene disrupted two different alleles of *Hydin.* Hy3 and OVE459 mice have identical pathophysiology to each other and to the spontaneous *hy3* mutant (Lawson and Raimondi, 1973; Johanson et al., 2001; Robinson et al., 2002; Davy and Robinson, 2003). *Hydin* is expressed specifically in ependymal cells of the ventricular system and the *Hydin* gene product may have a role in cilia function (Davy and Robinson, 2003).

Two other genetic mutations of interest are Musashi 1 and RFX4_v3 knockout mice. Musashi1 protein (*msi1*) is a RNA-binding protein found in ependymal cells and neuronal precursors. Homozygous *msi1-/-* mice have abnormal proliferation of ependymal cells surrounding the aqueduct of Sylvius, resulting in stenosis of the aqueduct and obstructive HC. Neuronal precursors appear relatively normal, suggesting that HC again results from an

ependymal cell defect (Sakakibara et al., 2002). Regulatory factor X4 variant 3 (RFX4_v3) is a transcription factor whose expression is restricted to the brain and regulates the expression of multiple genes critical for development, including Wnt pathway genes and BMP pathway genes (Zhang et al., 2007). Mice lacking a single allele fail to develop the subcomissural organ and have congenital HC. Removing both alleles causes severe midline malformations and death (Blackshear et al., 2003).

Pathology of hydrocephalus

While the exact cause(s) of HC remains unclear, the pathological changes associated with HC are fairly consistent and include physical, motor, cognitive, and behavioral abnormalities. The severity or extent of damage depends upon the age of onset, how quickly ventricles enlarge, and the magnitude of ventriculomegaly.

Loss of periventricular white matter. During the progression of HC, the periventricular white matter begins to atrophy and the corpus callosum and fornix become thinned (Kiefer et al., 1998; Del Bigio et al., 2003). The build-up of CSF in the ventricles causes stretching of the surrounding white matter, damaging the axons, myelin and blood vessels that it contains (Del Bigio, 2001), and cognitive deficits result when the deterioration of the fimbria/fornix connections (connecting the hippocampus to the forebrain) reach a threshold level of atrophy (Gadsdon et al., 1979; Egawa et al., 2002; Del Bigio et al., 2003; Del Bigio, 2004). Although stretching accounts for at least part of the white matter thinning, there is also a reduction in myelin content from the loss of axons and from impaired myelin production that begins prior to axonal damage (Del Bigio et al., 1997b; Del Bigio, 2000). Loss of oligogendrocytes in the

subependymal zone by apoptosois and necrosis could account for the decreased myelination (Del Bigio and Zhang, 1998). In chronic hydrocephalus increased CNPase activity (an oliogdendrocyte marker enriched in immature myelin) in periventricular white matter and increased expression of CGalT (indicative of myelin production) in the dorsal cerebrum myelin suggests that the turnover rate of myelin is increased (Del Bigio et al., 2003). Induction of HC in immature rats delays myelin production, but myelin can be restored if shunting is done before loss of axons occurs (Del Bigio et al., 1997c).

Loss of CSF flow**.** CSF contains many signaling molecules, growth factors, cytokines, and neurotransmitters that are present at specific times during development and under certain physiological conditions (Kasaian and Neet, 1989; Johnson et al., 1992b; Kitazawa and Tada, 1994; Nogi et al., 1997; Suzaki et al., 1997; Korhonen et al., 1998; Arnold et al., 1999; Ikeda et al., 1999; Riikonen et al., 1999; Grouzmann et al., 2000; Whalen et al., 2000). CSF normally circulates rapidly throughout the ventricular system and reaches most areas of the brain (Proescholdt et al., 2000), making CSF an important communication route. In HC, normal CSF circulation is lost, causing CSF to accumulate in the ventricles. Loss of CSF flow results in abnormal progenitor cell proliferation and accumulation of toxic waste products (Johnson et al., 1992a; Marin-Padilla, 1998; Nicholson, 1999; Mashayekhi et al., 2002). The neurological deficits that remain in cases of early onset HC, even after shunting, may be the result of improper CSF flow (McAllister and Chovan, 1998; Mashayekhi et al., 2002).

Grey matter atrophy. Thinning of the cortex is nearly always seen in both human and experimental cases of HC. While elevated intracranial pressure can cause cortical thinning and defects in progenitor cells are generally thought to be a consequence of HC, abnormal proliferation or differentiation of cortical neurons may also contribute to the pathology of HC. The germinal cells of the subventricular zone may not proliferate or migrate normally if hydrocephalus begins prenatally, impacting normal neuronal layering (Nojima et al., 1998; Khajeh et al., 2002; Owen-Lynch et al., 2003). One group reported that overexpressing cre recombinase in nestin-positive cells (neuronal precursors, radial glia and ependymal cells) resulted in genotoxicity, and the subsequent development of HC was due to impaired proliferation and death of neuronal precursors (Mignone et al., 2004; Forni et al., 2006).

To better understand the loss of grey matter Miyan et al 1998 studied the H-Tx rat at P0, 5, 10, 15, 20 and 25, using nucleic acid staining to compare the germinal matrix (subventricular zone), corpus callosum and frontal cortex of HC rats with normal littermates. Although present at birth, the corpus callosum and germinal matrix are reduced in HC rats at P5 and are nearly gone by P25, while both are still present in normal rats. The frontal cortex, while retaining laminar architecture, appears thinner at birth with a loss of space surrounding nuclei (the authors attribute this to loss of glial processes or to compression) and continues to shrink with time. Apoptosis does not seem to contribute to the loss as the number of apoptotic cells is actually decreased in HC rats in all three regions and at all timepoints. Early shunting of HC rats did not restore normal development, suggesting that elevated intracranial pressure was not responsible for the loss of gray matter (Miyan et al., 1998).

Altered trophic factor levels have been reported in early onset hydrocephalus and may be responsible for abnormal cortical development (Fukumitsu et al., 2000; Miyan et al.,

2001; Mashayekhi et al., 2002). Obstruction of the aqueduct occurs at P18 in the H-Tx rat, coinciding with the peak of neurogenesis and mass migration to the developing cortex; loss of subventricular zone neurogenesis and decreased migration is observed subsequent to aqueductal stenosis. The loss of normal CSF movement may prevent signals necessary for normal development from reaching the subventricular zone, resulting in a thinned cortex due to the loss of neuronal migration to the cortex and/or the loss of astrocytes and oligodenrocytes. (The cell populations primarily affected depends on age of onset. Neuronal progenitors are mostly generated prenatally while oligodendrocyte precursors are generated postnatally in the rat (Mashayekhi et al., 2002; Owen-Lynch et al., 2003; Khan et al., 2006). Progenitor cells apparently migrate prematurely in HC rats, resulting in fewer cells overall being produced in the germinal epithelium. The decrease in proliferation is likely due to factors (either the presence of an abnormal inhibitory factor, the lack of a normal growth factor, or the accumulation of normal growth factors to a concentration at which they become inhibitory) found in the CSF following obstruction of flow since progenitor cells from HC rats are able to proliferate *in vitro* when cultured with normal CSF. Progenitor cells from normal rats survive but do not proliferate *in vitro* in CSF from HC rats, suggesting that the HC CSF is not toxic. Intracranial pressure is not elevated in H-Tx rats and nor is apoptosis occurring, thus the loss of neurons due to decreased proliferation is likely the cause of cortical thinning (Jones, 1985; Miyan et al., 1998; Mashayekhi et al., 2002). Alternatively, Del Bigio et al (2003) found the cortex was thinner in early stages of chronic HC, but the total cerebral volume remained the same, suggesting that cerebral thinning is due mainly to stretching and not tissue loss (Del Bigio et al., 2003). They did not look to see if cell populations or water content were different, however.

Neuronal damage. Axonal damage in periventricular white matter is one of the earliest observations in HC. Periventricular axons are destroyed by mechanical injury from stretching, loss or decrease of cerebral blood flow, and the accumulation of waste products and toxins that accumulate in stagnant CSF (Del Bigio, 1993). Axonal degeneration correlates with ventricle enlargement and becomes more severe over time (Ding et al., 2001a, b). As the lateral ventricles enlarge with accumulating CSF, periventricular axons become stretched, activating L-type calcium channels in axons (Del Bigio, 2000; Johanson et al., 2001). Proteolytic calpains damage cytoskeletal proteins of axons in a calcium dependent manner and are partially responsible for the progressive loss of periventricular axons (Del Bigio, 2000).

Axon degeneration (as seen by silver impregnation in kaolin induced HC) occurs in layers IV-VI of the sensorimotor cortex, neostriatum, hippocampus, subiculum, fimbria, and corpus callosum. Stretching eventually causes disconnection of neurons in the striatum and cerebral cortex, and damage to these structures is associated with deficits in motor learning, memory, and nonverbal cognitive skills(Woody, 1986; Asanuma and Keller, 1991; van der Knaap et al., 1991; Fletcher et al., 1996b; Deiber et al., 1997; Shadmehr and Holcomb, 1997; van der Knaap et al., 1997; Del Bigio and Zhang, 1998). The corticospinal tract of the cervical spinal cord also is damaged in rats with chronic HC and is further evidence of damage to the motor cortex axons (Del Bigio, 2001; Del Bigio et al., 2003).

Disorganization and degeneration of thalamic neurons, subplate neurons (responsible for cerebral cortex and thalamus connections), and pyramidal neurons of the cerebral cortex is common in HC (McAllister et al., 1985; Robertson et al., 2000; Ulfig et al., 2001; Csillik

et al., 2002; Mori et al., 2002; Khan et al., 2006). Neurons, particularly in cortical layers V and VI, may have diminished capacity for activation in progressive HC. Reduced numbers of c-fos-immunoreactive cells and reduced c-fos protein expression is seen as early as P12 in H-Tx rats. Neuronal death does not appear to account for the reduction in c-fos since large numbers of normal-looking neurons are found in the areas of diminished c-fos expression (McAllister et al., 1999). Altered neuronal precursor proliferation and migration, ischemia and deafferentation may account for these changes (Oi et al., 1989).

While axonal damage is common, acute changes and neuronal death are rare in HC (Del Bigio and Zhang, 1998; Ding et al., 2001b, a; Del Bigio, 2004). However, there may be neuronal damage that is subtle but important to pathology (Del Bigio, 1993). Some investigators report finding "dark" neurons, swelling, and vacuolization in a small number of neurons in hippocampus, thalamus, caudate nucleus and in cortical and acetylcholinergic neurons (Gopinath et al., 1979; Wright et al., 1990; Hale et al., 1992; Ulfig, 2002). Other findings include neurofibrillary tangles on cortical and hippocampal neurons, altered expression of synaptic vesicle proteins, and loss of dendritic spines and branches (Ball, 1976; Ball and Vis, 1978; Fan and Pezeshkpour, 1987; Bret et al., 1990; Kriebel et al., 1993; Harris et al., 1996b; Del Bigio and Zhang, 1998; Del Bigio et al., 2003), which may explain the finding of impaired LTP generation in the hippocampus of HC rats (Katayama, 1992).

Ependymal denudation. The ependymal cell layer is lost in portions of the ventricular system in HC. Ependymal denudation is most frequently observed over white matter, particularly the dorsolateral angle and roof of the LV (Lawson and Raimondi, 1973; Page, 1975). In humans, there are case reports of normal, stretched, and completely denuded ependymal

layers throughout the ventricular system (Weller and Shulman, 1972; Bannister and Mundy, 1979; Del Bigio et al., 1985). Ependyma have a limited ability to proliferate, so as the ependymal cells are lost a glial scar usually forms in its place (Del Bigio, 1993). Remaining ependymal cells become flattened, lose their cuboidal shape, appear stretched and lose microvilli, (Price et al., 1976; Weller et al., 1978; James et al., 1980; Rascher et al., 1987; Kiefer et al., 1998). Ependymal denudation may contribute to the loss of directional CSF flow, resulting in stagnation and accumulation of CSF in the ventricles. The rate of ventricular enlargement may determine extent of ependymal loss (Collins, 1979), however as detailed previously there is substantial evidence for ependymal denudation prior to ventricular dilation.

Cerebral compression. Expansion of the ventricular system causes extracellular fluid and blood to be expelled from the brain in order to maintain a normal intracranial pressure (Hakim et al., 1976; Del Bigio, 1993). As extracellular space is compressed, diffusion of water and small molecules (including neurotransmitters) through the cerebral cortex is impinged (Nicholson and Sykova, 1998; Massicotte et al., 2000; Shoesmith et al., 2000; Sykova et al., 2001). Thus compression alters the extracellular environment in grey matter with unknown consequences. The reduced movement of fluids through the brain also causes stagnation in the large extracellular spaces of the periventricular white matter, which are then exposed to abnormally high concentrations of neurotransmitters and waste products found in the CSF of hydrocephalic patients (Lux et al., 1970; Del Bigio, 1989). The morphology of the blood vessels and vasculature is changed by compression, which could further reduce

blood flow and lead to ischemia (Epstein, 1952; Wozniak et al., 1975; Oka et al., 1985; Okuyama et al., 1987; Nakada et al., 1992; Ding et al., 2001a).

Altered cerebral vascularization and metabolism. Reduced cerebral blood flow (CBF) of up to 50% has been demonstrated in hydrocephalic humans and experimental models using PET, SPECT, MRI, CT and Doppler blood flow studies (Goh and Minns, 1995; Shih and Tasdemiroglu, 1995; Nakano et al., 1996). Capillaries are reduced in various experimental models and human cases (Gadson and Emery, 1976; Oka et al., 1985; Higashi et al., 1986; Okuyama et al., 1987; Del Bigio and Bruni, 1988; Sato et al., 1988; Jones et al., 1991; Nakada et al., 1992). In a study designed to compare capillary density in acute vs. chronic HC, Luciano et al (2001) found an initial decrease in cortical capillary density similar to previous studies. However, this acute decrease in capillaries was followed by recovery and increased density over a 12 week period. The functional significance of capillary recovery remains uncertain because cerebral blood flow is still reduced in chronic HC (Higashi et al., 1986; Luciano et al., 2001). HC may cause ischemic preconditioning that is neuroprotective in subsequent ischemic insults (Furuta et al., 1993; Barone et al., 1998; Ding et al., 2001a). This ischemic tolerance may in part explain why more neuronal death in not seen in HC.

Given the reduction in cerebral blood flow, it is not surprising that multiple groups have found changes in oxidative metabolism. During acute HC, increased lactate concentrations and lactate/pyruvate ratios in the frontal lobe, cortex and hippocampus are indicative of increased anaerobic metabolism, and hypoxic metabolites are found in the CSF from HC children (Del Bigio, 1989; Chumas et al., 1994; Braun et al., 1997; Hidaka et al., 1997; Braun et al., 1998; Braun et al., 1999; Socci et al., 1999; Braun et al., 2000; Massicotte

et al., 2000; Kondziella et al., 2002; Kawamata et al., 2003). Glucose metabolism also becomes impaired during HC. The CA3 region of the hippocampus is the first region to show such disturbances, which may contribute to problems with memory in HC patients (Kawamata et al., 2003). Magnetic resonance spectroscopy (MRS) studies with [1-^{13}C]glucose and [1,2-^{13}C]acetate injections in kaolin-induced HC rats show that multiple [1,2-^{13}C]acetate metabolites are significantly changed by week 4, while only one metabolite from [1-^{13}C]glucose is changed by week 6. Since acetate is only taken up by astrocytes and glucose is mostly taken up by neurons, it appears that astrocyte metabolism is primarily affected in HC (Kondziella et al., 2003). Additionally, transport of glutamine from astrocytes to neurons in the cerebrum is decreased, which could eventually lead to glutamatergic neurons losing function (Shank et al., 1985).

Changes in neurotransmitter concentrations and intracellular osmolytes are reported by many different groups, however there are considerable discrepancies between studies. Some studies found reduced glutamine, glutamate, aspartate, creatinine, N-acetyl-asprtate, taurine, alanine, dopamine, norpeinephrine, GABA, ATP and cAMP levels in H-Tx rats (Miwa et al., 1982; Higashi et al., 1986; Miyake et al., 1992; Minamikawa et al., 1994; Harris et al., 1997; Jones et al., 1997; Tashiro et al., 1997; Kondziella et al., 2002). In others, glutamine, glutamate, aspartate, GABA, norepinephrine, dopamine, noradrenaline, homovanillic acid and serotonin concentrations are increased (Miwa et al., 1982; Del Bigio et al., 1998; Kondziella et al., 2002). Differences in the ages of the animals and time after HC induction may account for some of the contradictory findings between studies.

Choroid plexus and circumventricular organs. Few morphological changes in the choroid plexus are documented in HC. Atrophied epithelial cells, microvilli distortion, vacuolization, intracellular inclusions and enlarged intercellular spaces are generally attributed to reduced production of CSF in an attempt to compensate for increased intracranial pressure (De, 1950; Hochwald et al., 1969; Dohrmann, 1971; Lawson and Raimondi, 1973; Go et al., 1976; Miyagami et al., 1976; Di Rocco et al., 1977; Collins and Woollam, 1979; Liszczak et al., 1984; Preston et al., 2003). More recently elevated cAMP in choroid epithelial cells has been documented in the *Tg737*^{orpk} mutant mouse (Banizs et al., 2005). As discussed previously, the subcommissural organ may be smaller, disorganized, or have reduced levels of glycoproteins. Reports of abnormalities in other circumventricular organs include reduced size and increased expression of angiotensin II receptors in the subfornical organ (Acikgoz et al., 1999; Somera and Jones, 2004).

Extracellular matrix. Increased depositions of fibronectin, laminin and vitronectin are seen in the rat model of PHVD and in human cases (Cherian et al., 2004b), particularly around blood vessels. The increased perivascular and parenchymal ECM may impede absorption of CSF, resulting in a build-up of CSF in the ventricles over time.

Changes in glia. A common reaction to CNS damage, astrogliosis is found in the perivascular and periventricular white matter in HC (Del Bigio, 1993; Takei and Sato, 1995), although whether gliosis is beneficial or harmful remains unclear. Elevated levels of GFAP and S-100b in the CSF of HC patients indicate irreversible loss of astrocytes and glia (since these are structural proteins not secreted by glia) (Albrechtsen et al., 1985; Tullberg et al.,

1998; Beems et al., 2003). However, increased subependymal gliosis and proliferation is higher in cats given an effective shunting procedure (compared to no shunt and ineffective shunt), even though the clinical outcome of effectively shunted animals was better (Takei and Sato, 1995). Astrocytes also proliferate and migrate to areas of ependymal denudation, forming a superficial layer. These cells express vimentin, normally only expressed by ependyma, which may indicate they have undergone differentiation (Wagner et al., 2003; Dominguez-Pinos et al., 2005).

Microglial activation in HC has yet to be fully understood. Activated microglia are the brain's macrophages, and increased numbers of activated microglia and clusters of phagocytic microglia were present in the periventricular white matter of P12 mild and severe HC rats and in the cortical grey matter of P21 HC rats (Mangano et al., 1998). Cells positive for the microglial marker mac-H were seen in older HC human fetuses (40 weeks) in the neuropil under areas without denudation and lying on the surface of denuded areas. However, younger fetuses did not have mac-H positive cells (Dominguez-Pinos et al., 2005) and no increases in microglia were found in rats with induced chronic HC (Del Bigio et al., 2003).

Cognitive deficits. The most common complication of HC in human patients is cognitive dysfunction (Young et al., 1973; Raimondi and Soare, 1974; Fletcher et al., 1992b; Fletcher et al., 1992a; Fletcher et al., 1996b; Fletcher et al., 1996a). 68% of shunted HC children have low IQ (Hoppe-Hirsch et al., 1998) and 72% of surviving premature birth infants with HC have "mental deficiencies" (Fernell et al., 1990; Fernell et al., 1991b). Cognitive impairment correlates to ventricle size. Damage to axons in the periventricular white matter

is believed to be the main cause of cognitive deficits (Del Bigio, 1993; Del Bigio et al., 2003; Khan et al., 2006), although abnormal development of some cortical neuronal populations may be responsible for some of the neurological impairments seen in HC children.

Impaired memory retention is another consequence of HC. Patients with chronic HC show signs of memory loss in adulthood (30's and 40's), perhaps caused by fluctuations is intracranial pressure and ventricle size over time (Hagberg and Sjorgen, 1966; Jones et al., 1995b; Scott et al., 1998; Dennis, 2000; Oi et al., 2000; Egawa et al., 2002; Khan et al., 2006). The hippocampus and septohippocampal cholinergic system (SHC) are believed to be critical for spatial learning and memory. Although only subtle changes have been observed in the hippocampus of HC brains, the axons of the SHC are located in structures that are damaged in HC, including the fimbria-fornix, hippocampal projections, and medial septum (Wenk, 1984; Toumane et al., 1988; Kriebel and McAllister, 2000). The alveus, fimbria and fornix are atrophied in humans and there are signs of axonal damage in these structures in HC rats (Del Bigio et al., 2003). Shim and colleagues (2003) found significant loss of cholinergic cells in the SHC pathway in kaolin-injected rats that correlated with severe deficits in learning and memory (assessed by morris water maze & passive avoidance tests to evaluate spatial learning and explicit memory, respectively). Both the loss of cholinergic neurons in the SHC pathway and behavioral deficits correlate with ventricle size, and deficits are worse in chronic HC than in acute HC(Shim et al., 2003). Shunting, if done early enough, can prevent memory deficits, arguing that shunting should be done even in cases where HC seems to have become arrested (Jones et al., 1995a).

Behavioral deficits. Autism and autism spectrum disorders (ASD) are more prevalent in children with HC than in the general population, affecting approximately 20% of HC children as compared to 0.3-0.6% of children without ventriculomegly (Fernell et al., 1991a, b; Chakrabarti and Fombonne, 2001; Kielinen et al., 2004; Lindquist et al., 2005; Persson et al., 2005; Lindquist et al., 2006). Other behavioral problems are common in children with HC. Unlike the general population, problems are reported equally in boys and girls (normally boys are reported to have more problems) (Connell and McConnel, 1981; Fernell et al., 1991a, b; Fletcher et al., 1995; Williams and Lyttle, 1998). The percentage of HC children with behavioral problems is as high as 60%, whereas only 3-5% of all school age children have reported issues (Rutter et al., 1976), and problems in HC children are reported to be extreme (Lindquist et al., 2006). Behavioral problems in HC children strongly correlate with the degree of cognitive impairment. 60-90% of children with IQ <70 have behavioral problems while 50-20% of children with IQ >70 have problems and only 12% of children with IQ>85 have problems. Preterm birth does not correlate with behavioral problems in HC children, although children with infantile HC are more likely to be hyperactive (Lindquist et al., 2006; Persson et al., 2006).

Motor dysfunction. Most children with HC have some form of motor disability, and gait disturbances are a common feature in adult onset or chronic hydrocephalus (McAllister and Chovan, 1998; Nowak and Topka, 2006; Bugalho and Guimaraes, 2007; Fraser and Fraser, 2007; Factora and Luciano, 2008; McGirt et al., 2008; Mori, 2008; Williams et al., 2008). 78% of premature surviving infants with HC have cerebral palsy and 60% of shunted HC children have motor deficits (Fernell et al., 1990; Hoppe-Hirsch et al., 1998). Like other

impairments caused by HC, motor deficits may correlate with ventricle size. Del Bigio et al (2003) report in rats with induced chronic HC, only animals with the greatest ventricle enlargement displayed motor deficits on the rotating cylinder. However, this was true only during the final (9 month) testing. HC rats also performed significantly more poorly during the 8 month time point but performance did not correlate to ventricle size. HC rats also tended to be less active and had significantly less rearing, but again ventricle size did not correlate with activity (Del Bigio et al., 2003). In the kaolin model, HC rats had readily observable differences in gait and posture but qualitative differences in performance on the rotating cylinder were minimal (Khan et al., 2006). Motor deficits may result from atrophy of the corpus callosum as severe thinning of the corpus callosum has been observed in animal models and humans. Stretching and/or disconnection of axons in the striatum or the cerebral cortex could also result in motor deficits (Gadsdon et al., 1979; Giroud and Dumas, 1995; Del Bigio et al., 2003).

Other impairments. Hormonal imbalances, seizures, sensitivity to sound and light, urinary incontinence, and visual deficits are seen with a higher than normal frequency in HC patients. Impaired depth perception and upward gaze/ocular movement is found in 24-30% of HC children (Zeiner et al., 1985; Hoppe-Hirsch et al., 1998) and 56% of surviving premature infants with HC have epilepsy (Fernell et al., 1990). Reduced body weight and short stature seen in some HC cases are likely due to hormonal imbalances and are possibly related to pituitary gland dysfunction or morphological abnormalities seen in hypothalamic nuclei (Del Bigio, 1993; Lopponen et al., 1996; Yoshino et al., 1999; Abdolvahabi et al., 2000). Rats with chronic HC gain less weight than controls even though they retain similar activity levels

(ie, they are physically able to eat) (Del Bigio et al., 2003). It is possible that stretching of the third ventricle affects the hypothalamus. If so, regulation of appetite or the pituitary gland could result in decreased weight gain.

Treatment of hydrocephalus

Diagnosis. The first diagnosis of hydrocephalus is credited to Hippocrates, who recognized that the enlarged head of some infants resulted from fluid accumulation in the brain and attempted to puncture the fontanelle (Davidoff and Chamlin, 1959). Today, macrocephaly (increasing head circumference) coupled with elevated intracranial pressure symptoms (nausea, bulging fontanelle, split sutures, poor feeding, irritability, headache, lethargy, vision impairment, declining developmental milestones) are indications of HC in infants (Vertinsky and Barnes, 2007). Ultrasound and CT (computed tomography) can detect enlarged ventricles and periventricular edema in fetuses and infants and are used in the initial diagnosis of HC to determine the need and urgency for surgical intervention. Ventricular enlargement is determined by taking the ratio of the diameter of the frontal horn to the maximum brain width (the Evans index). An Evans index greater than 0.3 is considered to be ventriculomegaly (Barkovich and Edwards, 1992; Vertinsky and Barnes, 2007). CT is also useful for follow-up evaluations on shunts and for determining shunt placement (Babcock et al 1988).

MRI (magnetic resonance imaging) has better resolution and contrast than CT and is also capable of detecting tumors that may be blocking the ventricular system. MRI uses no ionizing radiation, gives slice and 3D imaging, and shows circulation and CSF flow (Vertinsky and Barnes, 2007). MRI is used for obtaining anatomical detail for more

complicated surgeries (ie, to tell if CSF is moving through the aqueduct and to assist in catheter placement when cysts or isolated 4th ventricles are present) (Gammal et al., 1987; Quencer, 1992; Vertinsky and Barnes, 2007). MRI is also used to obtain detailed 3rd ventricle anatomy for endoscopic third ventriculostomy (ETV) surgeries and for determining ETV patency after surgery (Takahashi, 2006). SPECT (single photon emission computed tomography) can be used to evaluate cerebral blood flow and thus help determine if immediate surgical/medical intervention is required in children and adults with ventriculomegaly, but is not used as commonly as CT or MRI (Nayak et al., 2005).

In older children and adults, macrocephaly does not occur due to the hardening of the skull. Primary symptoms of HC in adults are gait disturbance, urinary incontinence, dementia, headaches, and vomiting (Hakim et al., 2001). Demonstration of enlarged ventricles by MRI or CT is used to confirm the diagnosis of HC. Gait disturbance presentation can vary, but typically presents as a slow, shuffling step characterized by a shortened stride, reduced foot-floor clearance and instability (Soelberg Sorensen et al., 1986). Frequent falls are common due to a loss of postural stability (Haan et al., 1987; Blomsterwall et al., 1995). Gait and stability frequently improve after shunt placement (Edwards et al., 2004). Urinary incontinence is due to an increased urgency coupled with difficulty reaching the bathroom because of gait disturbances (Ahlberg et al., 1988; Hakim et al., 2001). Cognitive impairments associated with HC are symptoms of frontal lobe impairment and include inattention, apathy, slowed responses, impaired executive functions, disinhibiton, forgetfulness, and excessive sleepiness (Rosen and Swigar, 1976; Masters and O'Grady, 1992; Lindqvist et al., 1993; Chemelli et al., 1999; Iddon et al., 1999; Hakim et al., 2001; Dauvilliers et al., 2003; Lindquist et al., 2005; Lindquist et al., 2008). Mild to moderate

cognitive deficits usually present later, after gait disturbances are manifest, and can fluctuate daily. Severe deficits or dementia that occurs prior to gait disturbances typically are not helped by shunting (Graff-Radford and Godersky, 1986; Iddon et al., 1999).

Surgical treatments. Left untreated, mortality rates of HC are 50-96% (Laurence and Coates, 1962; Yashon, 1963; Eckstein and Macnab, 1966). Surgical treatment of HC involves either redirecting CSF flow by shunt placement or creating a new pathway for CSF flow with endoscopic procedures. Currently shunt placement is the most widely used treatment for HC and is the most common neurosurgery in children (Hoffman and Smith, 1986; Pople et al., 1990; Beni-Adani et al., 2006). Shunts are used in cases of communicating HC and obstructive HC that includes an absorption problem. The first successful valved shunt surgery was described in 1952 by Nulsen and Spitz who diverted CSF into jugular vein. Currently CSF is diverted into the abdomen, lungs, or heart and the use of adjustable valves allows the drainage rate to be tailored to the individual as the rate needed varies widely among patients (Nulsen and Spitz, 1951; Takei and Sato, 1995; Zemack and Romner, 2002; Edwards et al., 2004).

Survival and outcomes of HC have greatly improved with use of shunts, although early placement, preventing severe damage, is best (Scarff, 1963; Takei and Sato, 1995; Del Bigio et al., 1997c). Brain weights and measurements of shunted and normal brains are comparable (Gadsdon et al., 1979). Ventricles become smaller, bulk flow of CSF is restored and edema of periventricular tissues is lost (Del Bigio et al., 1997a; Jones and Andersohn, 1998). Blood flow and metabolism improve, although capillaries generally do not regenerate (Wozniak et al., 1975; Del Bigio and Bruni, 1988; da Silva et al., 1994; da Silva et al., 1995;

Harris et al., 1996a; Nayak et al., 2005). Thinning of the corpus callusom and fornix is stopped, but few, if any, new oligodendrocytes are generated (Del Bigio et al., 2003; Fukushima et al., 2003). There are improvements in learning ability and behavior (Del Bigio et al., 1997a; Hawkins et al., 1997). In cases of chronic HC, 60-80% of patients given a shunt have clinical improvement as evidenced by a decrease in CSF outflow resistance and mean intracranial pressure (Vanneste, 2000; Petrella et al., 2008).

While shunt placement has greatly improved the life expectancy of HC patients, it also has the highest complication rate of all pediatric neurosurgeries (Renier et al., 1984; Walters et al., 1984; Oi and Matsumoto, 1985; Ammirati and Raimondi, 1987; Pople et al., 1990; Sainte-Rose et al., 1991; Pople, 1992; Bajpai et al., 1997; Baskin et al., 1998b). In the US, $100 million/year is spent on shunt procedures, and nearly half are revision surgeries (Bondurant and Jimenez, 1995). 70% of shunts fail within 10 years, and most children have multiple replacements, with a revision average of 1.6 to 3.6 revisions per patient (Tung et al., 1991; McCallum and Turbeville, 1994). Shunts can fail due to mechanical malfunction, infection, obstruction, migration, disconnection, or inadequate length for a growing child. Ventriculovascular shunts may develop clots in the catheter or blood vessel. Along with mechanical failure, infection is the most serious complication. Infection occurs in 2-39% of shunts (Yogev and Davis, 1980; Nelson et al., 1984; Blount et al., 1993). A majority (60%) of infections are caused by *Staphylococcus epidermidis* and *S.aureus* and 90% of infections occur within 6 months of placement, indicating that infection occurred at time of placement (Schoenbaum et al., 1975; Ersahin et al., 1994; Ronan et al., 1995; Turgut et al., 2005). Shunt obstruction is most common in cases of HC caused by tumors; other sources of obstruction include choroid plexus tissue, reactive glia, ependyma and cellular debris

(Lazareff et al., 1998). Overdrainage, slit ventricle syndrome, and craniosynostosis are also possible complications of shunts (Kang and Lee, 1999; Virella et al., 2002; Martinez-Lage et al., 2005; Daszkiewicz and Barszcz, 2007; Clark et al., 2008; Rekate, 2008).

In addition to surgical risks, shunt placement does not address all aspects of HC pathology. The ependymal layer does not regenerate (Del Bigio, 1993; Kiefer et al., 1998), and damaged axons generally cannot be restored (Del Bigio, 2001). There is some recovery in grey matter, but little repair of axons in white matter (Aoyama et al., 2006). Periventricular gliosis remains (Takei and Sato, 1995) and may contribute to the observation of stiffened ventricular walls, resulting in incompliance to changes in pressure (Kiefer et al., 1998). Additionally, the developmental consequences of removing or altering the composition of CSF are unknown.

Endoscopic procedures are used to treat obstructive, non-communicating HC by creating new pathways for CSF flow. Because they do not address problems involving reabsorption of CSF, endoscopic surgeries are generally not effective for treatment of communicating HC. Third ventriculostomy (ETV) is the most common endoscopic treatment and provides a bypass around the site of blockage by making an opening in the floor of the third ventricle. Success depends on the cause of HC. ETV are 70-85% successful in congenital HC cases with aqueductal stenosis or tumor, but are only successful in half of cases where HC is due to intraventricular hemorrhage or infection (Grant and McLone, 1997; Buxton et al., 1998; Buxton et al., 1999; Cinalli et al., 1999; Hopf et al., 1999; Fukuhara et al., 2002).

A successful ETV allows the patient to avoid shunt dependence and the accompanying complications as there is little to no risk of overdrainage, no foreign matter

left in the brain, no tubing to become blocked or fail, and low risk of infection. However, ETV have a high failure rate in infants (30-90% require shunts later) due to ongoing problems with CSF absorption. Success is better in older children, although the exact reasons are unclear (Baskin et al., 1998a; Cinalli et al., 1998; Cinalli et al., 1999; Siomin et al., 2001; Beems and Grotenhuis, 2002; Edwards and Pople, 2003; Rekate, 2004; Baykan et al., 2005; Dusick et al., 2008). Better classification of HC should help in determining which cases (ie, purely obstructive) would benefit from endoscopic treatment (Beni-Adani et al., 2006). Additionally, ventricular volume post ETV does decrease but remains higher than normal population, and there are risks of uncontrolled bleeding during operation and failure leading to death (Cinalli et al., 1999; Hader et al., 2002; Feng et al., 2004).

A new experimental treatment for preterm infants after intraventricular hemorrhage, called DRIFT (drainage, irrigation and fibrinolytic therapy), shows promise for preventing post-IVH HC. The theory behind DRIFT is to wash out as much blood, cytokines and free-radical causing agents (like excessive iron) from the ventricles as possible before ventricular dilation and HC can occur. Infants are also given plasminogen activator prior to irrigation with aCSF. A small pilot study was highly successful, but large-scale and long-term studies have yet to be completed (Cherian et al., 2004a).

Pharmacological treatments. There are no current pharmacologic therapies for HC, although there have been multiple attempts. Drugs designed to inhibit CSF production include acetazolamide, furosemide, isosorbide, and ouabain. Acetazolamide (Diamox) is a carbonic anhydrase inhibitor administered in the 1950's and reduces CSF production by as much as 60% *in vitro* and in experimental models. However there was no clinical improvement in HC

symptoms (Elvidge et al., 1957). Furosemide is a diuretic and acts by inhibiting renal sodium reabsorption. Again no improvement of HC symptoms was seen in a randomized drug trial (Shinnar et al., 1985; Witte et al., 1986; Kennedy et al., 2001; Whitelaw et al., 2001; Horinek et al., 2003). Isosorbide is a hyperosmolar agent that was promoted in the 1970's, but has not gone through randomized testing and lacks evidence for clinical efficacy (Lorber, 1975; Lorber et al., 1983; Del Bigio, 2004). Ouabain is a sodium-potassium ATPase pump inhibitor, but has widespread toxicity and thus is not suitable as a pharmacological treatment (Srebro, 1969; Gueli et al., 1976).

Agents designed to restore blocked CSF pathways also show little promise. Intrathecal injections of hyaluronidase did not show improvement (Schoeman et al., 1991). Urokinase, streptokinase, and tissue plasminogen activator were tested in hopes that they could break up blood clots following intraventricular hemorrhage, but had minimal success (Hansen et al., 1997a; Hansen et al., 1997b; Whitelaw, 2001; Whitelaw et al., 2001; Yapicioglu et al., 2003). Reducing inflammation of the menengies seems like a logical way to improve CSF absorption, but treatment with anti-inflammatory agents was also met with minimal success (Wilkinson et al., 1974; Erler and Klaber, 2001).

The most promising treatments for HC involve neuroprotective agents. Nimodipine is an L-type calcium channel antagonist that improves cerebral blood flow in models of ischemic injury (Scriabine and van den Kerckhoff, 1988; Scriabine et al., 1989; Schmidt et al., 1990; Welty and Horner, 1990; Feigin et al., 1998) and may help in HC by preventing the activation of calpains in axons that occurs during stretch injury, thus preventing proteolytic axonal damage (Maxwell et al., 1991; Povlishock and Jenkins, 1995; Stys, 1998; Buki et al., 1999; Del Bigio, 2000). In the kaolin rat model of HC, nimodipine treatment for 2 weeks

(started 2 weeks after induction of HC) prevented motor and cognitive deficits and preserved white matter (Del Bigio and Massicotte, 2001), although the myelin content and ventricle size did not differ from untreated controls. L-type calcium channels are also needed for synaptogenesis, so more work needs to be done to determine safe dosage levels (Vigers and Pfenninger, 1991; Kirsch and Betz, 1998; Del Bigio and Massicotte, 2001).

Magnesium sulfate, a calcium antagonist that blocks NMDA channel receptors and voltage and receptor-operated calcium channels, is another agent with neuroprotective potential. In experimental models of spinal chord injury, magnesium sulfate reduces lipid peroxidation, hypoxic-ischemic damage and the size of traumatic injury lesions (Simpson et al., 1994; Marinov et al., 1996; Suzer et al., 1999; Sameshima and Ikenoue, 2001; Vink et al., 2001). In the kaolin rat model (2 weeks of treatment given 2 weeks after induction), rats had reduced GFAP levels in the cerebrum but showed no improvement on the rotarod test, and higher doses produced significant sedation (Khan et al., 2003). Magnesium sulfate has been used in managing pregnant women with eclampsia, but recent studies show an association between its use and poor neurologic outcomes for the infants (Nelson and Grether, 1995; Mittendorf et al., 2002). Thus magnesium sulfate is not a viable HC treatment.

Mexiletine and riluzole are sodium channel blockers that showed some potential in various neurological injury models but also provided no protective effects in kaolin rat model of HC (Del Bigio et al., 2002).

Experimental hydrocephalus models

Induced models. Experimental models of HC have been created using a variety of substances injected into the ventricular system to block CSF flow. The first model of HC

involved inserting a cotton plug into the aqueduct of dogs (Dandy, 1919). Since then, many other agents have been used to induce HC, including blood (Bachs and Walker, 1953), India ink, cyanoacrylate glue, balloons, silicone oil, neuraminidase, infectious agents, and kaolin. Typically these are inserted or injected into the aqueduct, lateral, or fourth ventricle. Silicone oil is injected into the cisterna magna and produces a sterile mechanical obstruction to CSF out of the fourth ventricle (Wisniewski et al., 1969; Johnson et al., 1999). Neuraminidase is injected into the lateral ventricles and causes HC by cleaving surface sialic acid residues from the surface of ependymal cells, causing detachment and subsequent obliteration of the aqueduct (Grondona et al., 1996). Multiple agents have been used to cause meningitis and subsequent HC, including mycobacterium tuberculosis (De, 1950), influenza (Margolis and Kilham, 1977), mumps (Johnson and Johson, 1968), reovirus (Margolis and Kilham, 1969; Nielsen and Baringer, 1972), MHV3 mouse hepatitis virus 3 (Tardieu et al., 1982), and RS human respiratory syncytial virus (Lagace-Simard et al., 1982). In addition to inflammation, some of these agents also cause ependymal damage, denudation and aqueductal stenosis. A complication of most of these induced methods is the presence of an inflammatory response or physical tissue distortion that is not present in most cases of HC, thus the relevance of findings from models with inflammation is not clear.

Kaolin injection into the cisterna magna is an inexpensive and simple model of chronic noncommunicating hydrocephalus (Del Bigio and Zhang, 1998; Del Bigio et al., 2003; Khan et al., 2006). Kaolin causes sterile infmmation of the meninges, blocking the flow of CSF from the ventricular system to the intracranial and spinal subarachnoid space (the foramina of Luschka and Magendi are obstructed) (Kondziella et al., 2002). The development of HC takes place in several phases. The acute phase occurs after kaolin

injection and lasts for approximately four weeks and is characterized by maximal intracranial pressure and resistance to CSF outflow. After the fourth week, resistance and intracranial pressure drop, but ventricles continue to expand. This intermediate phase lasts 6-8 weeks, after which the model resembles chronic or normal pressure HC, although some believe it is most similar to postmeningitis HC in human infants (Oi et al., 1996; Kondziella et al., 2002).

Hydrocephalic rats have atrophy of periventricular white matter, reactive astrocytes, damaged neuronal axons and glial cell death. Deficits in motor and cognitive function are associated with damage to the corpus callosum, corticospinal tract and hippocampal fimbria/fornix projections. Severity of these deficits is related to the age of onset and the rate at which hydrocephalus progresses (Del Bigio and Zhang, 1998; Del Bigio et al., 2003). Severely HC rats have a learning disability in water maze. Early shunting can prevent this, while late shunting shows gradual but incomplete restoration of learning ability (Overholser et al., 1954; Del Bigio et al., 1997c).

HC can also be induced by altering nutrition. When female rats are fed a diet lacking in vitamins B12 or folic acid, they give birth to pups with HC. Embryos have stenosis of the aqueduct that begins between E16 and E18, with ventricle enlargement and HC beginning at E18 (Overholser et al., 1954; Newberne and O'Dell, 1959). Embryos from folic acid-deficient mothers also have loss of ependyma in the cerebral aqueduct.

Spontaneous models. Several spontaneously occurring congenital HC models are also commonly studied. One highly studied model is the H-Tx rat. Approximately 33% of H-Tx rats develop severe HC in late gestation following subcommissural organ abnormalities and obliteration of the cerebral aqueduct at E18 (Jones and Bucknall, 1988; Somera and Jones,

2004). Genetic studies show HC results from complex genetic factors with incomplete penetrance. Several loci for putative HC gene mutations have been identified, including one located near the gene *Igfb4* (insulin-like growth factor binding protein) (Jones et al., 2000). Approximately 15% of affected H-Tx rats have symptoms of HC after birth but live a normal life span. These are called hH-Tx rats, and are a model of compensated or spontaneously arrested HC. hH-Tx rats have a slow loss in learning ability as they age, similar to human cases of chronic HC (Kiefer et al., 1998; Nonaka et al., 2008).

Hyh mice are another spontaneous model that has been extensively researched. *Hyh* mice have a point mutation in the Napa gene encoding alpha-SNAP, causing a 40% reduction in protein levels (Chae et al., 2004; Hong et al., 2004). A four year study shows that 22.4% of hyh mice develop HC; 70% of these progress rapidly and die within 2 months while the rest survive up to 2 years (with no motor deficits and little to no enlargement of the head). The survivors develop spontaneous ventriculostomies that allow CSF to drain from the ventricles directly into the subarachnoid space (Batiz et al., 2006).

Other spontaneous models have been used in HC research but less is known about their pathology. In the SUMS/NP mouse, lateral ventricle enlargement begins on E15, the cerebral aqueduct is stenosed or obliterated prenatally, and ependymal development in the aqueduct is abnormal with dysfunctional cilia and discontinuous ependymal detachment. The cause for HC in this model has yet to be identified (Jones et al., 1987; Bruni et al., 1988a). The LEW/Jms rat also has aqueductal stenosis from unknown causes (Yamada et al., 1992). *Ch* mice have a point mutation in *Mf1 (*Foxc1), a forkhead/winged helix gene. The resulting mutant protein lacks its DNA-binding domain (Kume et al., 1998) and a defective chondroitin sulfate proteoglycan is produced (Green, 1970; Gruneberg and Wickramaratne,

1974; Richardson, 1985). Hydrocephalus begins on E12, possibly due to abnormal subarachnoid space or loss of ependymal cells since the cerebral aqueduct remains patent (Green, 1970; Gruneberg and Wickramaratne, 1974). In WIC-Hyd rats an unknown genetic mutation causes cilia dysfunction and HC (Torikata et al., 1991; Nakamura and Sato, 1993). Other spontaneous HC models include *hop sterile* (hop) mice and *obstructive hydrocephalus (oh)* mice.

Transgenic models. Numerous lines of transgenic animals develop HC for a variety of known and unknown reasons. HC develops in two separate lines of mice lacking the L1 neural adhesion molecule, and severity ranges from mild ventriculomegly to severe HC (Dahme et al., 1997; Fransen et al., 1998). The aqueduct is patent at early stages of HC but becomes stenosed as HC progresses (Rolf et al., 2001). Hypoplasia of the corticospinal tract is a consistent finding and mirrors the human CRASH syndrome (Fransen et al., 1995; Cohen et al., 1998; Fransen et al., 1998). A mutation in hydin, a cilia protein expressed exclusively in ventricular ependymal cells, causes HC in *Hy3* mice (Davy and Robinson, 2003). Ependymal denudation precedes stenosis of the aqueduct, which occurs late in the pathogenesis (Raimondi et al., 1976; Jimenez et al., 2001).

Not all transgenic models have aqueductal stenosis. Two lines of mice over-expressing TGF- β1 in GFAP positive cells develop progressive communicating HC and have increased depositions of perivascular extracellular matrix proteins (Galbreath et al., 1995; Wyss-Coray et al., 1995; Cohen et al., 1999), reduced LTP in hippocampal slices, and impaired spatial learning in Morris water maze (Gerlai et al., 1995). The conditional knockout of *Cnp1* in oligodendrocytes results in neuronal degeneration in white matter, HC

and early death (Lappe-Siefke et al., 2003). Interestingly, myelin and myelination are normal, which shows that oligodendrocytes have axonal support functions beyond myelination. The function of CNP (2',3'-cyclic nucleotide 3'-phosphodiesterase) in oligodendrocytes is unclear, but it can interact with cytoskeletal proteins (Laezza et al., 1997; Bifulco et al., 2002). Both full knockouts and mice with reduced expression of nonmuscle myosin heavy chain II-B (NMHC-B, involved in cell polarity, cell shape, cell motility, cytokinesis) results in HC. Severity correlates with the level of gene expression. Ependymal cells denude and the aqueduct is malformed, likely due to weak cell-cell interactions of neuroepithelial cells and possibly resulting in improper differentiation. There are defects in neural cell migration as well (Uren et al., 2000). The conditional knockout of *Hif-1α* in nestin expressing (neural) cells results in HC and impaired memory (Tomita et al., 2003). HIF-1α is a hypoxia-inducible factor involved in cell survival during ischemia, and also has role in vascular development. Null mutants of *Spag6, Hfh4, Pol λ, Polaris, Mdnah5* all have cilia dysfunction and HC (Chen et al., 1998; Taulman et al., 2001; Ibanez-Tallon et al., 2002; Kobayashi et al., 2002; Sapiro et al., 2002).

Hydrocephalus has long been recognized as a serious neurological disorder. Modern advances in imaging techniques and biomedical technology has made a tremendous impact in the treatment of patients with the disorder. Surgical treatments have provided a greatly prolonged life expectancy for persons with HC, but their quality of life could be improved if pharmacological treatments for reversing or preventing HC were available. As summarized above, HC has a complicated etiology and past attempts for drug therapies were only marginally successful at best. Experimental HC models have been useful in identifying common features of HC, including abnormalities in ependymal cells, cilia, the

subcommissural organ, and neuronal progenitor cells. Yet the question of cause and effect remains for many of these mechanisms, and the current experimental models have not been able to effectively address these issues. Many models cannot predict which animals will develop HC, making the distinction between early causative events and pathological responses very difficult. Other models are complicated by the presence of pathologies distinct from HC, again making the identification of the molecular mechanisms of HC tricky. Further advances in HC treatment will be aided by a model that allows for control of HC onset and accurate prediction of affected animals.

CHAPTER 3
A NEW GENETIC MODEL OF HYDROCEPHALUS

We developed a transgenic mouse line that expresses the G_i-coupled RASSL (Receptor Activated Solely by Synthetic Ligand) Ro1 in GFAP-positive cells with the aim of studying the role of GPCR signaling in astrocyte-neuronal communication. Surprisingly, we found that all transgenic mice expressing Ro1 developed hydrocephalus. We analyzed these mice in an effort to develop a new model of hydrocephalus that will further our understanding of the pathophysiology of the disease.

Expression of Ro1 was restricted to glia by crossing the transgenic hGFAP-tTA (tet transactivator behind the human glial fibrillary acidic protein promoter) mouse line with the transgenic tetO-Ro1/tetO-LacZ mouse line. This cross produced double transgenic mice that expressed Ro1 only in glia. All double transgenic mice developed hydrocephalus by p15, while single transgenic littermate controls appeared normal. Hydrocephalic double tg mice displayed enlarged ventricles, partial denudation of the ependymal cell layer, altered subcommissural organ morphology, and obliteration of the cerebral aqueduct. Severely hydrocephalic mice also had increased levels of phosphoErk and GFAP expression.

Administration of doxycycline to breeding pairs suppressed Ro1 expression and the onset of hydrocephalus in double transgenic offspring. Double transgenic mice maintained on dox never developed hydrocephalus, however if they were taken off doxycycline at weaning, double transgenic mice developed enlarged ventricles within seven weeks,

indicating that Ro1 expression also induces hydrocephalus in adults. Signaling through Ro1 is required for hydrocephalus to occur since both ventricular enlargement and ependymal denudation were prevented by administering a KOR inverse agonist to double transgenic mice taken off doxycycline. This study discovered a new model of hydrocephalus in which the rate of pathogenesis can be controlled, enabling the study of the pathogenesis of both juvenile and adult onset hydrocephalus.

Materials and methods

Animals. All experiments were performed in accordance with guidelines set forth by the Institutional Animal Care and Use Committee at the University of North Carolina at Chapel Hill and federal guidelines. Animals were maintained in climate-controlled housing with a 12 h light/dark cycle and were given food and water *ad libitum*. The day of birth was defined as postnatal day 0 (P0).

Transgenic mouse lines. The B6(C3)-Tg(GFAP-tTA)96Kdmc mouse line was made by isolating the tTA gene from pUHD 15-1 (kindly provided by Hermann Bujard, Universität Heidelberg, Heidelberg, Germany), and cloning it downstream of the 2 kb human GFAP promoter in place of lacZ in the pGfa2lac1 vector (kindly provided by Michael Brenner, University of Alabama at Birmingham). The GFAP-tTA-mP1 cassette was placed between four copies of genomic insulators from chicken ß-globin gene (Chung et al., 1993). Transgenic mice were obtained by standard methods and backcrossed to C57BL/6J for at least five generations. FVB/N-TgN(tetO-Ro1-LacZ)CONK mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and crossed to GFAP-tTA mice to get double-

transgenic mice. KOR knock-out mice (Hough et al., 2000) were obtained courtesy of Dr. John E. Pintar (University of Medicine and Dentistry New Jersey, Piscataway, NJ).

Genotyping. Polymerase chain reaction (PCR) was used to screen mice for transgene presence. For B6(C3)-Tg(GFAP-tTA) transline, the following primers were used: 5' CCCTTGGAATTGACGAGTACGGTG 3' (forward) and 5' TGGTGTATGAGCGGCGGCGACGGCAG 3' (reverse). For FVB/N-TgN(tetO-Ro1-LacZ)CONK transline, primers against the LacZ gene were utilized: 5' ATCCTCTGCATGGTCAGGTC 3' (forward) and 5' CGTGGCCTGATTCATTCC 3' (reverse).

Southern blots. To identify KOR KO mice, genomic DNA was digested with BamHI overnight at 37ºC. DNA fragments were separated on a 0.8% agarose gel (95 volts for 3.5 hours) and transferred to Zeta-Probe GT membrane (Bio-Rad Laboratories, Hercules, CA) using an alkaline transfer method. DNA was immobilized by UV crosslinking. The ^{32}P-labelled hybridization probe was made using the Megaprime^{TM} DNA labeling kit (Amersham Life Science, Buckinghamshire, England) and 100 ng of the 0.8 kb XbaI/EcoRI fragment of kappa genomic clone from plasmid GC#3 (kindly provided by John Pintar, University of Medicine and Dentistry New Jersey, Piscataway, NJ). The membrane was hybridized in QuikHyb buffer (Stratagene Cloning Systems, La Jolla, CA) and exposed to Kodak Biomax Film overnight at -70° C.

Doxycycline administration. Selected animals were given 25 or 50 µg/ml doxycyline hyclate (dox; Sigma, St. Louis, MO) in the drinking water. Amber bottles wrapped in aluminum foil were used to protect the dox from light and the water was changed once a week. Breeding

pairs on dox were given dox water at the time of mating and maintained on dox continuously. Breeding pairs off dox were never exposed to dox. For timed studies, mice were taken off dox at P30.

Spiradoline administration. Spiradoline mesylate (0.6 µg/µL in ddH_2O, U-62066, Sigma, St. Louis, MO) was given to mice (3 mg/kg, i.p.) 15-30 minutes before behavioral testing or euthanizing.

NorBinaltorphimine administration. nor-Binaltorphimine dihydrochloride (Tocris, St. Louis, MO) was suspended in PBS to make a 1 mM solution and aliquots were stored at -20ºC. Mice were injected with norBIN (20 mg/kg, s.c.) every three days, starting with the day doxycycline was removed from the drinking water. For behavioral tests, mice were injected with norBIN 18-24 hours prior to testing.

Immunofluorescence. Adult (P45–P60) mice were deeply anesthetized and transcardially perfused with cold 4% paraformaldehyde/0.1 M $NaPO_4$, pH 7.4. Brains were removed and postfixed overnight at 4°C in the same buffer. To detect the Ro1 receptor, sections were probed for FLAG, the peptide (DYKDDDDK) epitope tag fused to the N terminus of Ro1. For FLAG staining, 50 µm sections were cut on a vibratome, washed in alternating PBS and PBS/0.5% Triton X-100 (Tx-100) washes, and blocked in PBS/0.5% Tx-100/10% NGS/0.2% gelatin/3% milk. Sections were incubated with primary antibodies (rabbit anti-FLAG; Sigma) diluted in PBS/0.5% Tx-100/3% NGS/0.2% gelatin overnight at 4°C with gentle agitation. Sections were extensively washed in PBS and PBS/0.2% Tx-100. Secondary antibodies (goat anti-rabbit IgG conjugated to Alexa-488; Invitrogen, Carlsbad, CA) were diluted in PBS/0.5% Tx-100/5% NGS/0.2% gelatin and applied for 2–4 h at room temperature. For

GFAP and NeuN staining, fixed brains were cryoprotected in PBS/30% sucrose overnight at 4°C. Sections (14 µm) were cut on a cryostat, blocked for 2–4 h in PBS/20% normal goat serum/2% BSA/0.2% Tx-100 at room temperature, and incubated overnight with primary antibodies (mouse anti-GFAP, 1:500, Sigma; rabbit anti-GFAP, 1:500, Roche, Mannheim, Germany; mouse anti-NeuN, 1:1000, Chemicon, Temecula, CA; rabbit anti-ERK1/2 and anti-phosphoERK1/2, Cell Signaling, Beverly, MA; mouse anti-acetylated tubulin, Sigma, St. Louis, MO; mouse anti-S100β, Sigma, St. Louis, MO; anti-cleaved caspase 3, Cell Signaling, Beverly, MA) diluted in the same buffer. After washing, sections were incubated with secondary antibodies (goat anti-mouse conjugated to Alexa-488 and goat anti-rabbit conjugated to Alexa-594; Invitrogen) diluted 1:400 in blocking buffer for 2–4 h at room temperature.

Ependymal cell dissection. Adult mice were anesthetized and euthanized by decapitation. Coronal sections (400 µm) containing the lateral ventricles (usually four sections) were cut on a vibratome (Leica VT 1000S; Leica, Nussloch, Germany). Using a dissecting microscope, fine point microdissection tweezers (World Precision Instruments, Sarasota, FL) and 30 gauge needles, the ependymal cell layer(s) were teased away from the underlying tissue.

Xgal histochemistry. Adult mice (P30–P45; $n = 6$) were deeply anesthetized and transcardially perfused with cold 4% paraformaldehyde/0.1 M $NaPO_4$, pH 7.4. One hundred micrometer sections were cut on a vibratome. Sections were washed three times (10 min each) at room temperature in Xgal rinse buffer (0.1 M KPO_4, pH 7.4, 2 mM $MgCl_2$, 0.01% sodium deoxycholate, 0.2% Igepal CA-630) and stained overnight at 37°C in Xgal stain

buffer [rinse buffer plus 1 mg/ml 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside, 5 mM $K_3Fe(CN)_6$, and 5 mM $K_4Fe(CN)_6$].

Immunoprecipitation/Western blotting. Fresh brain tissue (P21–P50; *n* = 15) was homogenized in ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM EDTA, 50 mM NaF, 10 mM NaPP, 200 µM Na_3VO_4, 1% Tx-100, 100 µg/ml PMSF, 1 µg/ml leupeptin, 2 µg/ml aprotinin, 1 µg/ml soybean trypsin inhibitor, 1 µg/mlpepstatin A, 10 µg/ml benzamidine) and sonicated on ice. Lysates were incubated on a rotator for 1 h at 4°C. Supernatants were precleared and incubated for 2 h at 4°C with M2 FLAG antibody (Sigma) and protein A/Sepharose beads (Sigma) that had been blocked with BSA. Beads were boiled in Laemmli buffer, run on an SDS-PAGE gel, and blotted onto a nitrocellulose membrane. The blots were blocked in 5% milk–TBS/0.1% Tween 20 (TBST) for 2 h at room temperature and probed overnight at 4°C with a 1:250 dilution of a rabbit anti-FLAG polyclonal antibody (Sigma) in 5% milk/TBST. Membranes were washed in TBST, probed with HRP-conjugated goat anti-rabbit IgG (1:10,000), and processed for ECL. For GFAP, fresh brains (P21–P35; *n* = 6) were dissected in ice-cold PBS, and tissues were homogenized in a volume of homogenization buffer (50 mM HEPES, pH 7.3, 150 mM $NaCl_2$, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 1% Tx-100 with Complete protease inhibitor (Roche Diagnostics, Mannheim, Germany) equal to 10x tissue weight. The samples were sonicated and centrifuged. Equal amounts of protein were run on a Tris-glycine gel, blotted onto a nitrocellulose membrane, and probed with rabbit anti-GFAP antibody (DakoCytomation; 1:5000) for 48 h at 4°C.

Hematoxylin and eosin staining. Brains from perfused animals (P30–P66; *n* = 14) were fixed in 10% formalin overnight, rinsed in distilled water, and stored in 70% ethanol. The fixed brains were taken to the University of North Carolina histology core for paraffin embedding. Sections (6 µm) were cut on a sliding microtome. Sections were incubated in xylenes to remove paraffin, rehydrated in serial ethanol washes, and incubated for 6 minutes in hematoxylin (Sigma, St. Louis, MO). After rinses in 0.25 acid alcohol and lithium carbonate, sections were counterstained in eosin Y solution (Sigma, St. Louis MO), dehydrated in serial ethanol washes, cleared in xylenes, and mounted in Cytoseal™ XYL (Richard-Allan Scientific, Kalamazoo, MI).

Immunocytochemistry. Brains from adult mice (P30–P45; *n* = 11) were fixed in 4% paraformaldehyde, cryoprotected in PBS/30% sucrose, and cut in 35 µm sections on a cryostat. Sections were rehydrated in PBS and incubated in 100% methanol at –20°C for 10 min. Sections were washed three times (10 min each) in TBST and once in TBS before incubating for 2 h in blocking buffer (5.5% NGS/5.5% BSA/TBST) at room temperature. Avidin/biotin was blocked using an avidin/biotin blocking kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's protocol. Primary antibodies [rabbit anti-phospho-p44/42 mitogen-activated protein (MAP) kinase (Thr202/Tyr204); Cell Signaling Technology, Beverly, MA; 1:250; or mouse anti-GFAP; Sigma; 1:500] were diluted in blocking buffer, and sections were incubated overnight at 4°C. Biotinylated goat anti-rabbit secondary antibody (Vectastain Elite ABC kit; Vector Laboratories) was diluted 1:500 in TBS/3% BSA, and sections were incubated for 2 h at room temperature. Sections were then incubated for exactly 30 min in 0.6% H_2O_2 at room temperature. ABC reagent was prepared according to kit instructions, and sections were incubated for 2 h at room temperature. DAB

reagent (Vector Laboratories) was added, and the reaction was allowed to proceed until sufficient staining developed. Coverslips were mounted using Vectashield with DAPI (4',6'-diamidino-2-phenylindole) (Vector Laboratories).

Ventricle size. Brains fixed in 4% paraformaldehyde were embedded in agarose and cut coronally into 500 µm (P15; n = 22) or 375 µm (P0; n = 24) sections on a vibratome (Leica VT 1000S; Leica, Nussloch, Germany). Sequential sections were viewed under a light microscope (Zeiss, Oberkochen, Germany), and images were collected using MetaMorph imaging software. The section at the level of striatum and anterior commissure was selected from each animal, and the size (maximum width) of the lateral ventricles was measured using the MetaMorph software and divided by the maximum brain width. The resulting ratio of ventricle to brain diameter provides a measurement of hydrocephalus severity (Jones et al., 2001a). Hydrocephalic phenotypes were classified as mild (0.20–0.40), moderate (0.41–0.60), or severe (>0.60).

RNA purification. Ependymal cell dissections (as described above) were done in RNAlater (Ambion, Foster City, CA) using RNase-free instruments. Total RNA was purified using the RNeasy Lipid Mini Kit (Qiagen, Germantown, MD) according to the manufacturer's protocol. RNA was eluted in a final volume of 60 µL RNase-free water containing SUPERase·In™ (1 U/µL, Ambion, Foster City, CA) and concentrated using a Savant SpeedVac Concentrator (Thermo Scientific, Waltham, MA). RNA concentration was determined using NanoDrop™ 1000 spectrophotometer (Thermo Scientific, Waltham, MA) and samples were sent to the University of North Carolina Genomic and Bioinformatics core

facility for RNA quality analysis. Samples with a quality score less than 7.0 were discarded. Samples were stored at -20º C if not used immediately.

Gene microarrays. Affymetrix Mouse GeneChip Gene 1.0ST microarrays were used to detect changes in gene expression between Ro1 double transgenic mice and single transgenic littermate controls at 5 (n=3) and 9 (n=4) days after dox removal. 0.5 to 1.0 ug of total RNA from each animal was sent to the University of North Carolina Functional Genomics core facility. Each sample was amplified to obtain 7 μg of total RNA that was used to synthesize cDNA. A custom cDNA kit from Life Technologies was used with a T7-$(dT)_{24}$ primer for this reaction. Biotinylated cRNA was then generated from the cDNA reaction using the BioArray High Yield RNA Transcript Kit. The cRNA was then fragmented in fragmentation buffer (5X fragmentation buffer: 200mM Tris-acetate, pH8.1, 500mM KOAc, 150mM MgOAc) at 94^{o}C for 35 minutes before the chip hybridization. 15 μg of fragmented cRNA was then added to a hybridization cocktail (0.05 μg/μl fragmented cRNA, 50 pM control oligonucleotide B2, *BioB*, *BioC*, *BioD,* and *cre* hybridization controls, 0.1 mg/ml herring sperm DNA, 0.5 mg/ml acetylated BSA, 100mM MES, 1M [Na^{+}], 20mM EDTA, 0.01% Tween 20). 10 μg of cRNA was used for hybridization. Arrays were hybridized for 16 hours at 45^{o}C in the GeneChip Hybridization Oven 640. The arrays were washed and stained with R-phycoerythrin streptavidin in the GeneChip Fluidics Station 400. After this, the arrays were scanned with the Hewlett Packard GeneArray Scanner. Affymetrix GeneChip Microarray Suite 5.0 software was used for washing, scanning, and basic analysis. Sample quality was assessed by examination of 3' to 5' intensity ratios of certain genes.

Microarray data analysis. CEL and CHP files were sent to Expression Analysis, Inc. (Durham, NC) for two-group comparison with permutation analysis for differential expression (PADE). As no genes were found to be differentially expressed with a false discovery rate (FDR) less than 1, genes with a fold change $\geq .25$ and a p-value ≤ 0.5 (as determined by student's T-test assuming equal variance) were selected for pathway analysis. The DAVID bioinformatics database (http://david.abcc.ncifcrf.gov/home.jsp, National Institute of Allergy and Infectious Diseases, NIH) was used for gene-annotation enrichment analysis, KEGG pathway and BioCarta pathway mapping. Gene lists and expression data were sent to GeneGo Bioinformatics Software, Inc. (St. Joseph, MI) for detailed pathway analysis using the MetaCore platform.

Real time PCR. RNA (2.5-3.0 µg total RNA) was converted to cDNA using random primers (250 ng/µL) and SuperScript™ II reverse transcriptase (Invitrogen, Carlsbad, CA) in 20 µL total volume following the manufacturer's protocol. PCR was carried out using 1 µL cDNA for genes of interest or 1 µL of a 1:10 cDNA dilution for the GAPD reference gene and 20X TaqMan® gene expression assay primer/probe sets (Applied Biosystems, Foster City, CA) in a 20 µL final volume. Reactions were run in triplicate on a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) using the default PCR thermal cycling conditions. The reporter dye signal (FAM) was normalized using the passive reference dye, ROX, to eliminate volume-handling error. Fold change was determined using the comparative C_T method ($\Delta\Delta C_T$).

Astrocyte cell culture. Litters were collected 0-2 days postnatal and lab protocol was followed to obtain sections of cortex from the pups. DNA preparations from the tails were screened for the presence of the tTA and lacZ genes by PCR. The cortex cell suspensions from individual pups were plated in 12 well plates, one pup per well. After seven days RASSL/tTA positive cultures were combined and cultures of wild-type or tTA only cells were combined. The cells were then plated either in 24 well plates for cAMP assays or on polylysine-coated 15 mm circle coverslips.

cAMP assay. 24-well plate cultures were incubated in 0.8 uCi ^{3}H-Adenine per well for two hours. 0.2 mM IBMX was added to block the breakdown of cAMP. Cultures were then incubated in plain media, isoproterenol (1 uM), clonidine (10 uM), spiradoline (1 uM), isoproterenol and clonidine, or isoproteranl and spiradoline for fifteen minutes. Cells were lysed in 5% cold TCA. ATP and cAMP were eluted using Dowex and Alumina columns. Radioactivity of both elutions was measured in a scintillation counter and the data normalized for cell population.

CHAPTER 4
CHARACTERIZATION OF THE RO1 HYDROCEPHALUS MODEL

Glial cells, the majority of which are astrocytes, carry out numerous functions critical for the central nervous system to develop and operate normally. Certain of these functions are passive and supportive in nature. Astrocytes envelope neurons and contact blood vessels, providing metabolic and nutritive support. Astrocytes prevent neuronal excitotoxicity by buffering extracelluar potassium and removing excess neurotransmitters from synapses (Gabriel et al., 1998; Walz, 2000; D'Ambrosio et al., 2002). During development glia are critical for normal migration of neuronal precurors. Recent studies suggest astrocytes can actively modulate or initiate neuronal signaling (Walz, 1989; Keyser and Pellmar, 1994; Vernadakis, 1996; Carmignoto et al., 1997; Pasti et al., 1997; Araque et al., 1999; Araque et al., 2000; Araque et al., 2001; Pasti et al., 2001; Araque et al., 2002; Fiacco and McCarthy,

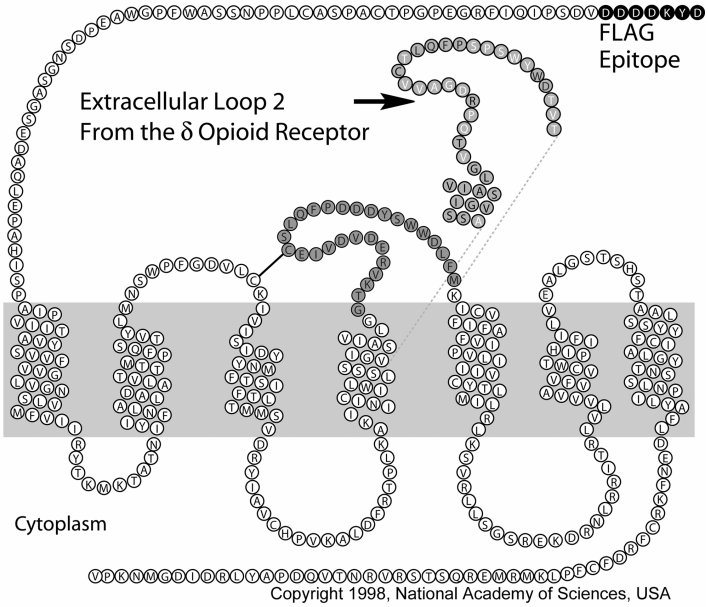

Figure 5. The Ro1 receptor. To create Ro1, the second extracellular loop of the κ-opioid receptor was replaced with the second extracellular loop of the δ-opioid receptor. A FLAG tag was added to the N terminus for detection. Ro1 has a greatly reduced affinity for endogenous ligands but still responds to the small molecule drug spiradoline.

2004). If astrocytes actively regulate neurophysiology, alterations in astrocyte-neuronal signaling could contribute to the etiology of many CNS disorders. However, isolating astrocytic signaling systems to test this hypothesis is difficult since astrocytes and neurons express a similar complement of neuroligand receptors (Porter and McCarthy, 1996). Consequently, neuronal responses to astrocyte signaling under normal or pathological conditions have been difficult to measure directly.

We generated a mouse line expressing the G_i-coupled Ro1 RASSL (Receptor

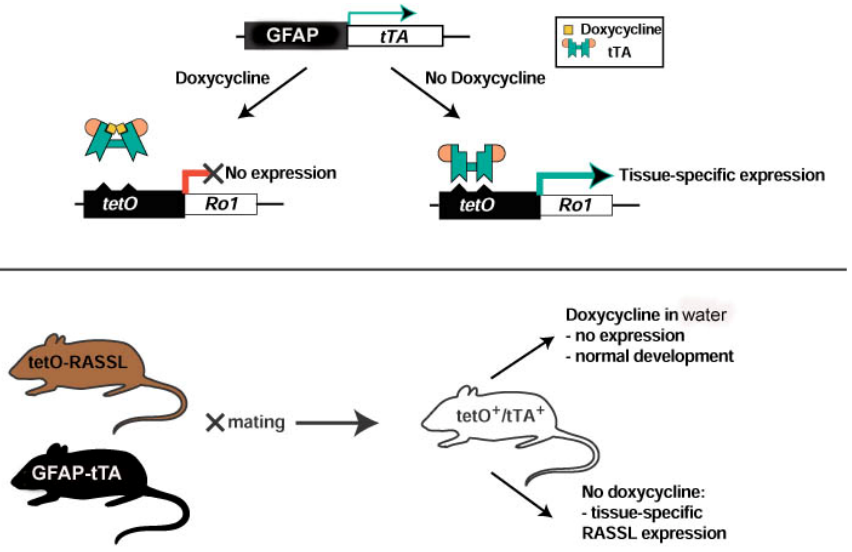

Figure 6. Tetracycline inducible system. Tissue-specific Ro1 expression in GFAP-tTA$^{+/-}$ :: tetO-Ro1$^{+/-}$ double transgenic mice is induced by the removal of doxycycline. Tissue specificity is achieved by crossing GFAP-tTA mice with tetO-Ro1 mice; double-transgenic offspring will have Ro1 expression in cells expressing GFAP (top). Doxycycline represses Ro1 expression by binding tTA. Removal of doxycycline induces Ro1 expression (bottom).

Activated Solely by Synthetic Ligand) in glial fibrillary acidic protein (GFAP)-positive cells to directly assess the role of astrocytic G-protein coupled receptors (GPCRs) in neurophysiology. Ro1 is a kappa opioid receptor (KOR) modified by replacing its second extracellular loop with the delta opioid receptor second extracellular loop (Figure 5) and adding the FLAG® epitope tag to the N terminus. Conklin and colleagues demonstrated that Ro1 has a 200-fold decrease in binding affinity for dynorphin, the endogenous ligand for

KOR. Ro1 also has significantly reduced signaling in response to multiple dynorphin and enkephalin peptides while retaining its ability to bind spiradoline and *nor*binaltorphimine, a small molecule agonist and inverse agonist, respectively (Coward et al., 1998; Redfern et al., 1999). This is possible since the endogenous peptides bind to the KOR second extracellular loop while small molecule drugs bind in transmembrane regions of the receptor (Schwartz, 1994). Expressing Ro1 in glia on a KOR knockout background allows us to activate G_i-coupled receptor signaling in glia while measuring changes in neuronal excitability.

Restricting Ro1 expression to GFAP-positive cells required crossing two lines of transgenic mice, the Ro1 line and the tet-transactivator (tTA) line. The Ro1 line carries the Ro1 gene under the control of the tetO promoter; the tTA line carries the tTA gene under the control of a 2.0 kB fragment of the human glial fibrillary acidic protein (hGFAP) promoter. In order for the Ro1 receptor to be expressed, tTA must bind the tetO promoter and initiate transcription. Since tTA expression is restricted to GFAP-positive cells, double transgenic progeny will only express Ro1 in glia. The timing and level of Ro1 expression in double transgenic mice can be manipulated with doxycycline. Doxycycline (dox) prevents Ro1 expression by binding to

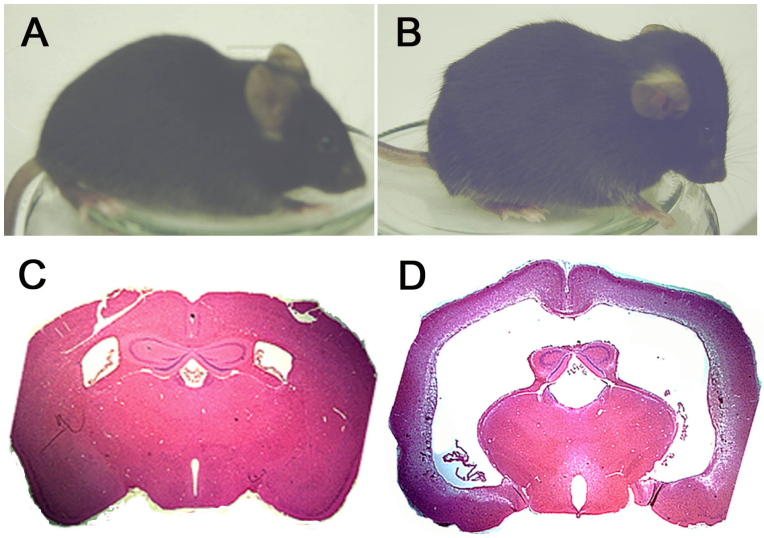

Figure 7. Double transgenic mice off dox develop hydrocephalus. Double transgenic mice on dox (A,C) appear normal while double transgenic mic off dox display megalocephaly (B) and enlarged lateral ventricles (D).

tTA, thus preventing tTA from binding to the tetO promoter (Figure 6). Dox is able to cross the blood brain barrier, so Ro1 expression is turned off by maintaining transgenic mice on dox water and turned on by removing dox from the drinking water.

Crossing Ro1 and tTA transgenic lines produced double transgenic (tg) mice in expected numbers. However, the double tg mice unexpectedly developed severe hydrocephalus when maintained off dox (Figure 7). This is the first indication that activating a G_i–coupled GPCR in GFAP-positive cells can lead to hydrocephalus. G_i–linked signaling is involved in various physiological processes including proliferation, chemotaxis, hormone secretion, modulation of neurotransmission and contraction of cardiomyocytes (Pace et al., 1991; Spiegel, 1992b, a; Wickman and Clapham, 1995; Jan and Jan, 1997). The classical G_i signaling pathway is the inhibition of adenylate cyclase resulting in a decrease of cAMP production, and it is now recognized that G_i signals via the ERK/MAPK pathway as well. Although how altering G_i–linked signaling cascades in glia results in HC is unclear, the prevalence of GPCRs and their importance in the CNS makes it unsurprising that changes in GPCR cascades causes pathology.

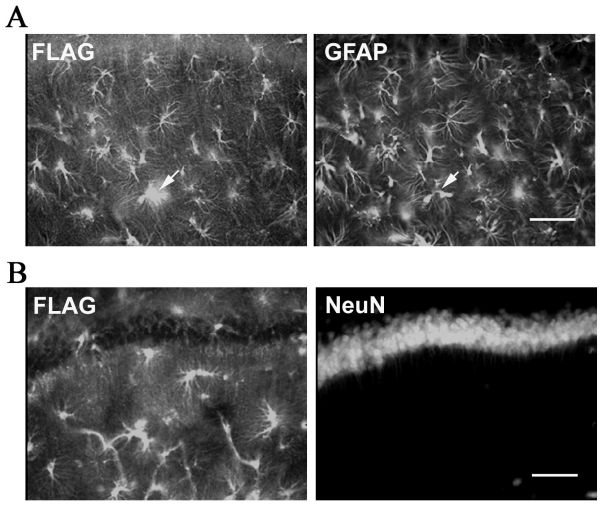

Figure 8. Ro1 expression is restricted to astrocytes and is regulated by dox. (***A***,***B***) Hippocampal brain sections were double-stained for FLAG and GFAP (astrocyte marker) or FLAG and NeuN (neuronal marker). (***A***) Positive FLAG staining (left, arrow) colocalized with GFAP (right, arrow) but not with NeuN (***B***). Arrows point to the same cell. Scale bars: ***A,B*** 100 µm

Results

Expression of Ro1. Mice expressing Ro1 in GFAP-positive cells were generated by crossing the hGFAP-tTA mouse line to the tetO-Ro1/tetO-LacZ mouse line. This genotype was moved onto a KOR knock-out background (Hough et al., 2000) to prevent spiradoline from activating endogenous kappa opioid receptors. $KOR^{-/-}$ /hGFAP-$tTA^{+/-}$ or $KOR^{-/-}$/hGFAP-$tTA^{+/+}$ mice were crossed to $KOR^{-/-}$/tetO-Ro1/tetO-$LacZ^{+/-}$ mice to produce double tg (hGFAP-$tTA^{+/-}$ /tetORo1/tetO-$LacZ^{+/-}$) mice, single tg (hGFAP-$tTA^{+/-}$ or tetO-Ro1/tetO-$LacZ^{+/-}$) mice, and mice with neither transgene, all on the KOR background. Double tg mice were produced in expected numbers and are indistinguishable from littermates at birth. To determine that Ro1 expression is restricted to glia, brain sections from double tg mice were probed with anti-FLAG (the epitope tag fused to Ro1) antibodies plus either anti-GFAP (Glial Fibrillary Acidic Protein, an astrocytic marker) or anti-NeuN (neuronal nuclei, a neuron-specific nuclear protein) antibodies. Positive FLAG staining is apparent in most brain regions, including cortex, hippocampus, cerebellum and thalamus. FLAG staining is seen in GFAP-positive cells but not in NeuNpositive cells (Figure 8A and 8B), confirming that Ro1 expression is restricted to glial cells.

Since Ro1 expression is driven by the tetO system, dox can be used to manipulate when Ro1 is expressed. To verify that dox represses Ro1

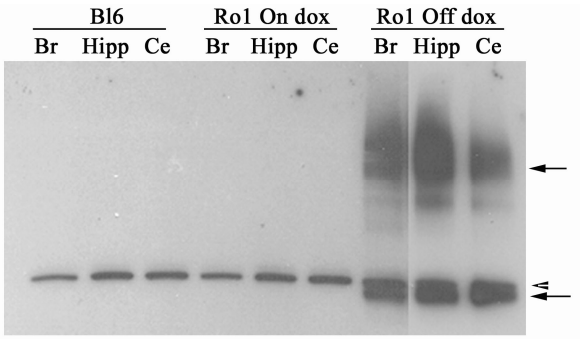

Figure 9. Ro1 expression is controlled by doxycycline. Brain lysates from a C57/Bl6 control mouse, Ro1 mouse on dox (25 µg/mL), and Ro1 mouse off dox were immunoprecipitated with FLAG monoclonal antibody and the blots probed with FLAG polyclonal antibody. Only the Ro1 mouse off dox had positive bands (solid arrows). A non-specific band was found in all samples (arrowhead). Br=brain, Hipp=hippocampus, Ce=cerebellum.

expression, brain tissue extracts from double tg animals (P60) that had been maintained off dox, double tg animals that had been maintained on 25 μg/mL dox and C57Bl/6J wild-type control animals were immunoprecipitated with a monoclonal Flag antibody. A band at approximately 44 kDa, consistent with the predicted size of the receptor, along with bands representing multimers of the receptor, were detected in extracts from double tg animals maintained off dox. No FLAG-specific bands were detected in wild type mice or double tg mice on 25 μg/mL dox, indicating that 25 μg/mL dox represses Ro1 expression (Figure 9). Since Ro1 tg mice also carry a tetO-LacZ transgene, we used Xgal histochemistry to confirm that dox regulates expression (Figure 10). No Xgal staining was detected in any brain region of sections from double tg mice on 25 μg/mL dox. Strong Xgal staining was observed in the hippocampus, thalamus and brainstem of double tg mice maintained off dox while weak Xgal staining was seen in the cortex.

Characterization of hydrocephalus. Although double tg mice *off dox* appear grossly normal at birth, by postnatal day 15 (P15) 100% of double tg mice off dox begin to exhibit macrocephaly characterized by a swollen, dome-shaped cranium (Figure 11B). By 12 weeks of age all double transgenic mice off dox develop severe hydrocephalus and approximately 50% die (Figure 11C). Survival studies were terminated at 12 weeks due to the severe morbidity of the remaining double tg animals. Dissection of the brain revealed greatly enlarged ventricles filled with fluid, indicative of hydrocephalus and impaired CSF homeostasis. Blood on the surface of the brain was frequently observed in hydrocephalic mice and on occasion excess CSF was seen between the skull and brain surface. Control single tg littermates (hGFAPtTA$^{+/-}$ only or tetO-Ro1/tetO-lacZ $^{+/-}$ only) on or off dox and

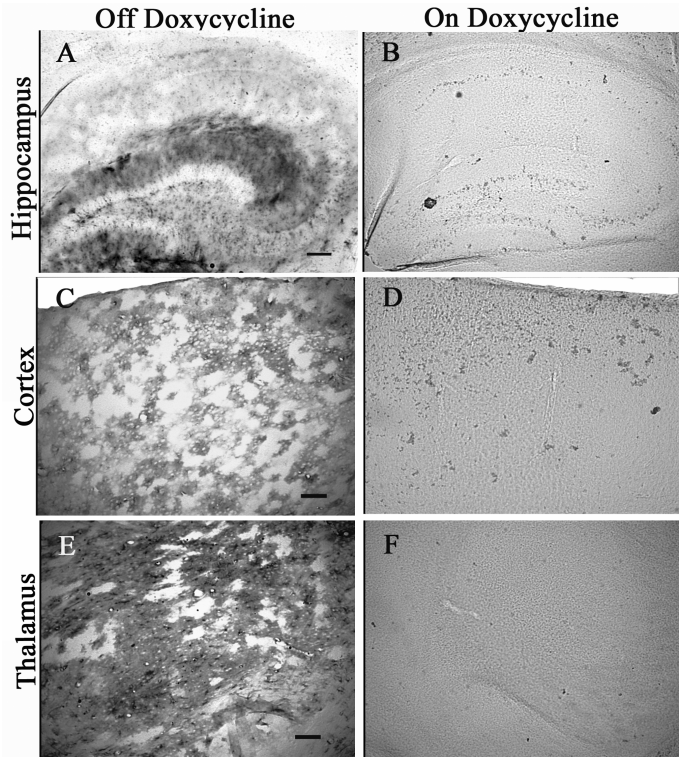

Figure 10. LacZ expression is regulated by dox. Brain sections from Ro1 mice on dox (25 µg/ml) (***B,D,F***) and Ro1 mice off dox (***A,C,E***) were stained by Xgal histochemistry. Ro1 mice on dox had no detectable positive cells, whereas robust staining was seen in Ro1 animals off dox, indicating that dox is effectively controlling dox expression. Scale bars: ***A.B*** 150 µm; ***C-F*** 50µm.

double tg mice on 25 or 50 µg/mL dox appear normal at P15 and do not develop macrocephaly at any age, indicating that the insertion of either or both transgenes does not cause hydrocephalus.

To further characterize the progression of hydrocephalus in double tg mice, the ventricle size of litters *off dox* were examined at P0 and P15 (Figure 12). At P0 double tg mice (n=10) and control littermates (n=14) showed no enlargement of the lateral ventricles. At P15, however, all double tg mice (n=12) exhibited enlarged ventricles with a ventricle to brain ratio greater than 0.30 while control littermates (n=10) had an average ratio of 0.10.

We observed variation in the severity of the hydrocephalus phenotype (Table 1) as defined in Materials and

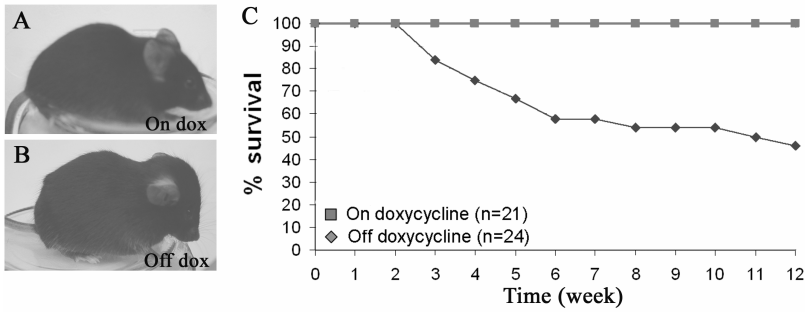

Figure 11. Ro1 mice maintained off dox develop hydrocephalus. All Ro1 pups from breeding pairs maintained off dox ***(B)*** develop overt hydrocephalus; more than 50% die by 12 weeks of age (***C***). Ro1 mice maintained on dox (25 µg/mL) appear normal (***A***).

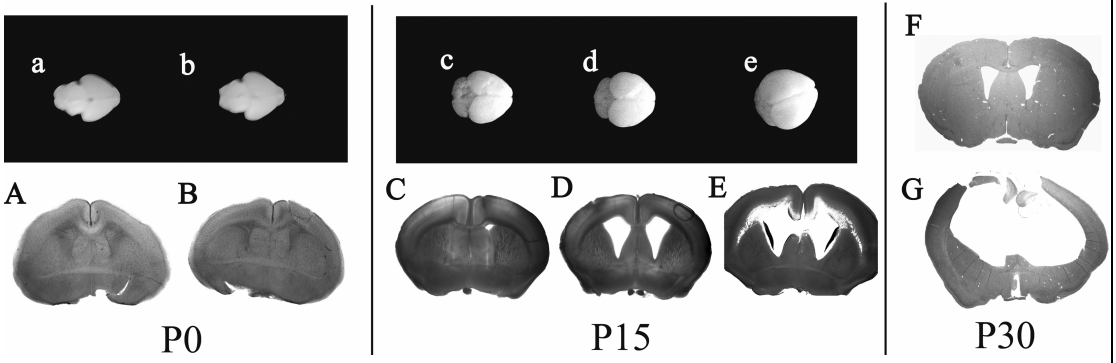

Figure 12. Ro1 mice develop progressive enlargement of the lateral ventricles. At birth, whole brains and coronal sections of Ro1 mice (***b,B***) and single transgenic control littermates (***a,A***) are indistinguishable. By p15, brains from Ro1 mice (***d,e,D,E***) are larger than controls (***c,C***) and have pronounced enlargement of the lateral ventricles. The hydrocephalus in Ro1 mice can be classified as mild (***D,*** n=4), moderate (***E,*** n=6) or severe (not shown, n=2) cases based on ventricle to brain ratio. By p30 Ro1 mice (***G***) have severe hydrocephalus characterized by greatly enlarged lateral ventricles and extremely thin cortex. Control mice at p30 (***F***) appear normal. All mice were maintained off dox.

Methods. The severity of hydrocephalus is not linked to homozygosity as all animals measured were heterozygous for both transgenes. The severity of hydrocephalus continued to progress with age since all double tg animals off dox developed severe hydrocephalus by P30 (Figure 12*G* and author's observations).

Brains of four control mice (single transgenic, P30 to P125) and five

Table 1. Severity of hydrocephalic phenotype in P15 Ro1 mice

Phenotype	*Average ratio*	n
Mild	0.31	4
Moderate	0.50	6
Severe	0.67	2

hydrocephalic double tg (P30 to P68) mice off dox were serially sectioned and stained with hematoxylin and eosin for a more detailed histological analysis. The most striking feature of the hydrocephalic brains was the greatly enlarged lateral ventricles (Figure 13*G*). The third ventricle, optic recess, pineal recess and intraventricular foramen were also enlarged. The

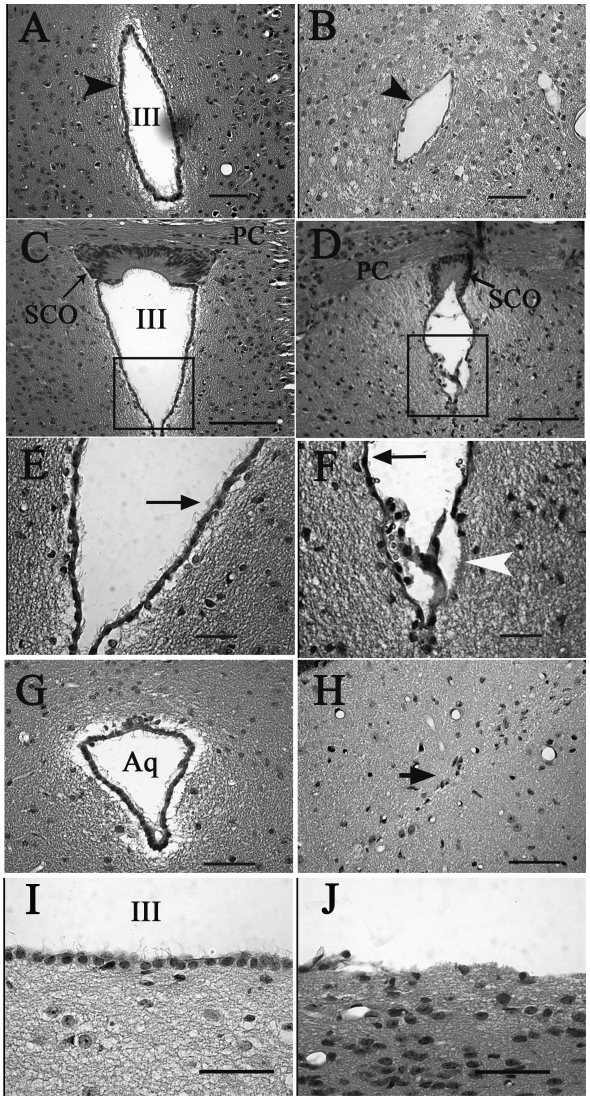

Figure 13. Alterations in the ventricular system of hydrocephalic Ro1 mice. Hematoxylin and eosin staining of coronal sections form single-transgenic control (P45, left column) and Ro1 (P33, right column) mice. ***A,B,*** In Ro1 mice (***B***), the third ventricle at the level of the periventricular hypothalamic nucleus is reduced in size and the ependymal cell layer appears thinner compared with controls (***A***). ***D,F,H,J,*** The subcommissural organ is disorganized in Ro1 mice (***D***), and there is a partial denudation of ependymal cells lining the ventricular walls (***F***, arrowhead; ***J***). The remaining ependymal cells have fewer cilia (***F,*** arrow), and the aqueduct is obliterated with no apparent ependymal cell layer (***H***). ***C,E,G,I,*** In control mice, the subcommissural organ (***C***) and aqueduct (***G***) show normal morphology. The ependymal layer is intact with multiple cilia per cell (***E***, arrow; ***I***). ***E*** and ***F*** are enlargements of the boxes in ***C*** and ***D***, respectively. Mice were maintained off dox. Aq=aqueduct, SCO=subcommissural organ, III=third ventricle, PC=posterior commissure. Scale bars: ***A,B,G,H,I,J,*** 50 µm; ***C,D,*** 100 µm; ***E,F,*** 30 µm.

cerebral cortex was reduced in thickness, with the most severely affected animals exhibiting an almost completely atrophied cortex. All cortical layers appeared to be present (data not shown). The septum and retrosplenial cortex-hippocampus transition were disrupted; other brain regions, including the hippocampus, appeared compressed and caudally displaced. Red blood cells indicative of recent hemorrhaging were found throughout the brain (data not shown).

The white tract tissue underlying the lateral ventricles was disrupted and there was an incomplete denudation of ependymal cells lining the lateral (data not shown) and third ventricles (Figure 13*J*). In some instances the ependymal layer appears to be separated from the underlying tissue (Figure 13*F*). While it is possible that the separation is due to a fixation artifact, ependymal layer

separation was not observed in control mice.

Where intact, the ependymal cell layer appeared thinner (Figure 13*B,D,F)*, the ependymal cells appeared flattened and had lost their cuboidal shape, and not all ependymal cells were ciliated. The rostral end of the aqueduct of Sylvius was completely obliterated (Figure 13*H*), suggesting that hydrocephalus results in part from the blocked flow of cerebral spinal fluid between the third and fourth ventricles. No ependymal cells were detected at the site of obstruction and the surrounding tissues appeared vacuolated. Abnormalities in the subcommissural organ (SCO) have been implicated in other models of hydrocephalus (Jones and Bucknall, 1988; Perez-Figares et al., 2001; Blackshear et al., 2003; Fernandez-Llebrez et al., 2004; Krebs et al., 2004); likewise in double tg brains the SCO appeared disorganized (Fig. 13*D*).

Adult onset of hydrocephalus. To determine if hydrocephalus results from Ro1 expression during development, mice were kept on 25 μg/mL dox until weaning (P21) and maintained off dox until P60. Due to hardening of the skull, none of the double tg mice taken off dox developed overt enlargement of the cranium by P60; however double tg mice (n=4) had enlarged lateral ventricles with an average ventricle to brain ratio of 0.30. By comparison, the ventricle to brain ratio for littermate controls (n=2) was 0.11, similar to that of double tg mice (n=8, ratio=0.15) and controls (n=7, ratio=0.13)

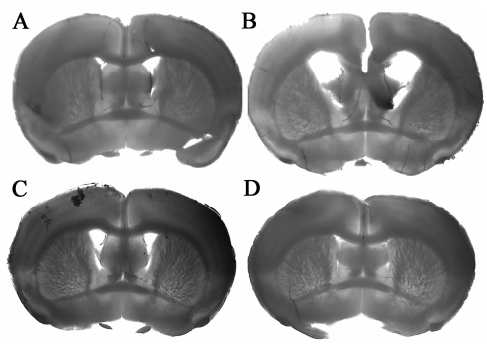

Figure 14. Ro1 mice will develop enlarged ventricles if taken off dox at weaning. (A,B). In litters taken off dox at weaning (p21), Ro1 mice (***B***) develop enlarged ventricles when compared to single transgenic littermate controls (***A***). (***C,D***) Ro1 mice (***D***) have normal ventricles when mice were kept on dox continuously as compared to single transgenic littermate controls (***C***). All mice were processed at p60. A, *n = 2;* B, *n* = 4; C, *n* = 7; D, *n = 8.*

that were maintained on dox continuously until P60 (Figure 14). Hydrocephalus appears to develop with Ro1 expression independent of age and thus is not dependent on processes linked to early stages of development.

Inhibition of Ro1 signaling. Since overexpression of a transgene may cause abnormalities due to disruption of normal cell transcription and translation machinery, signaling through Ro1 was suppressed with *nor*Binaltorphimine (*nor*BNI), a long-acting KOR inverse agonist (Jones and Holtzman, 1992; Ko et al., 1999; Holtzman, 2000). Double tg mice and single tg littermate controls were divided into three treatment groups (Figure 15). In the first group, double tg (n=2) and controls (n=2) were kept on 50 µg/mL dox. In the second group, double tg (n=4) and controls (n=4) were taken off 50 µg/mL dox at P30. In the third group, double tg (n=6) and controls (n=5) were taken off 50 µg/mL dox at P30 and given injections of *nor*BNI (20 mg/kg, s.c.) every three days. All groups were sacrificed at P60 and ventricle to brain ratio

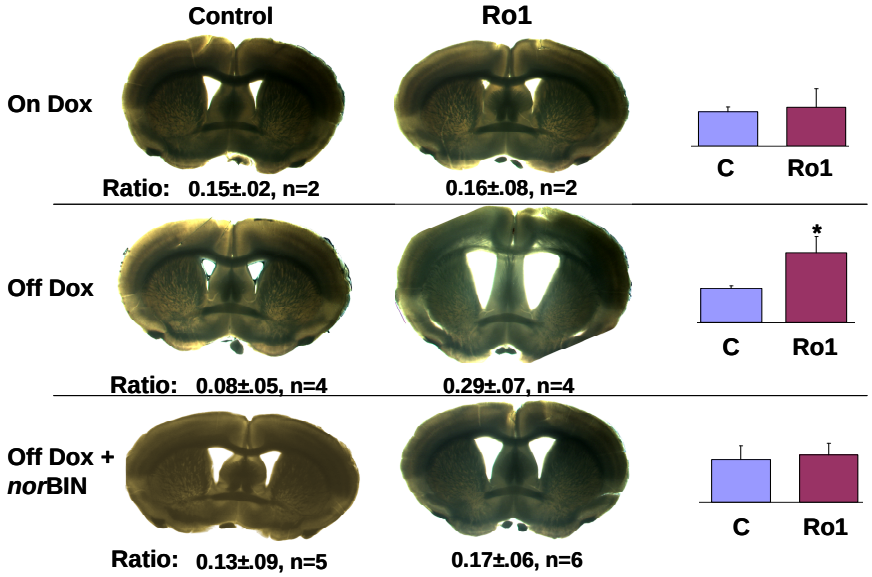

Figure 15. Blocking Ro1 intrinsic activity prevents ventricle enlargement. Ro1 mice and littermate controls were taken off dox at P30 and injected with the KOR antagonist *nor*BNI (7.6 mg/kg i.p.) every three days for 30 days. The ratio of lateral ventricle diameter to brain diameter (ratio) was measured in fixed sections at the level of striatum and anterior commissure. Ratios are significantly higher (p=0.02) in Ro1 mice off dox than in controls (ratios from controls on and off dox were combined for statistics). There is no significant difference in ratios between controls and Ro1 mice on dox or off dox plus *nor*BNI.

measured. As expected, no significant difference was found between double tg (0.16±0.08) and littermate single tg (0.15±0.02) controls on dox, while double tg mice taken off dox had a significantly larger ratio (0.29±0.07) than control mice (0.08±0.05). There was no difference in the ratios of double (0.17±0.06) and single (0.13±0.09) tg mice taken off dox and given *nor*BNI. Since expression of the receptor alone did not result in enlarged ventricles, signaling through Ro1 appears to be required for HC to develop.

Blocking Ro1 signaling also prevented ependymal denudation in double tg mice. The same treatment groups listed above were sacrificed 10 days after dox removal and their brains processed for H and E staining. Only the double tg mice taken off dox showed loss of ependymal cells in the lateral ventricles, while *nor*BNI treated double tg mice and double tg mice on dox had intact ependymal cell layers similar to controls (Figure 16).

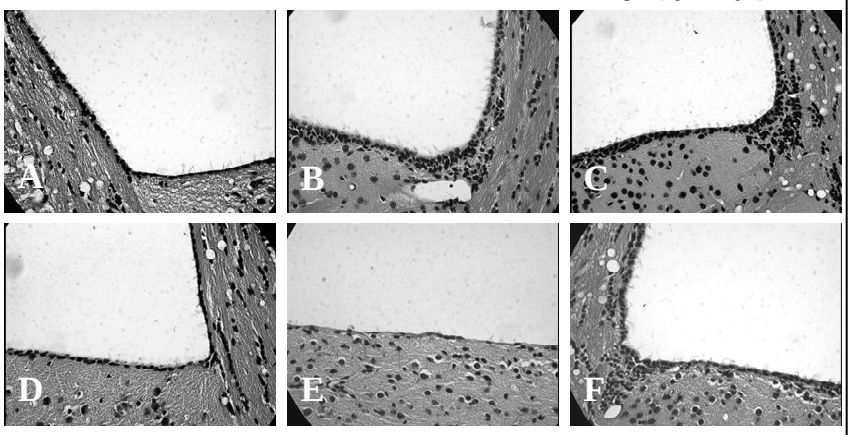

Figure 16. Blocking Ro1 signaling prevents ependymal denudation. Ro1 mice (***D,E,F,*** n=2) and littermate controls (***A,B,C,*** n=2) were kept on dox, taken off dox, or taken off dox and given *nor*BNI at P30 and sacrificed 30 days later. Both control (***A***) and Ro1 (***D***) mice kept on dox have normal ependyma. Control mice taken off dox have normal ependyma (***B***) while ependymal denudation is evident in Ro1 mice (***E***) taken off dox. Both control and Ro1 mice given *nor*BNI after dox removal have normal ependyma. All sections are from the medial wall of the lateral ventricle at the level of the anterior commissure and striatum.

<u>GFAP and phosphoErk</u>. Increased GFAP expression, an indicator of reactive glia, is observed in most forms of damage to the brain, including hydrocephalus. Increased levels of GFAP staining were seen in hydrocephalic Ro1 mice. The cortex, a region typically low in

GFAP immunoreactivity, and the hippocampus showed the greatest increases in GFAP staining compared to control mice (Figure 17). Western blots confirmed that GFAP protein levels were elevated in the cortex and hippocampus of Ro1 mice; GFAP levels in cerebellar tissue extracts from the same mice appeared similar to controls (Figure 17A).

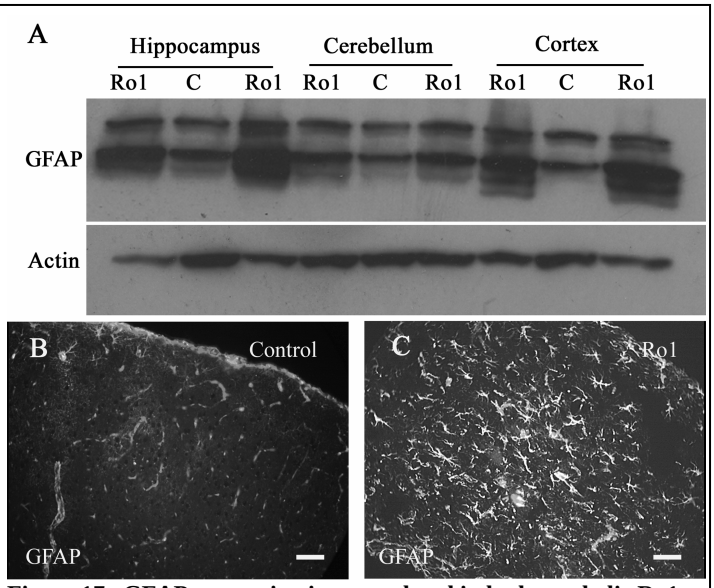

Figure 17. GFAP expression is upregulated in hydrocephalic Ro1 mice. Brain lysates from two Ro1 mice with severe hydrocephalus and one littermate control (P22) were separated by SDS-PAGE and probed with anti-GFAP antibody (***A***). Ro1 mice have increased GFAP levels in the hippocampus and cortex. Brain sections from control (***B***) and Ro1 (***C***) mice were stained for GFAP to show increased expression. Cortical sections are shown; pial surface is on the top right corner. Scale bar, 50 µm.

Because G_i-coupled receptors are known to activate the mitogen-activated protein (MAP) kinase pathway, coronal sections from Ro1 mice were stained for phosphorylated extracellular signalregulated kinase (Erk) p44/42, the active form of Erk p44/42. Increased phosphoErk staining was observed in the superficial layers of entorhinal cortex and in the striatum surrounding the lateral ventricles of Ro1 animals off dox, but not in littermate single transgenic controls (Figure 18).

Discussion

To investigate how G_i protein signaling in astrocytes affects brain neurophysiology, we expressed the RASSL Ro1, a G_i-coupled GPCR, in GFAP-positive using the hGFAP promoter and the tet-off system. Unexpectedly, all mice expressing Ro1 developed non-

communicating triventricular hydrocephalus. Hydrocephalus appears to result from Ro1 expression since single transgenic mice do not develop hydrocephalus as would be expected if it was caused by an insertional defect. Hydrocephalus is prevented by suppressing Ro1 expression with dox and by blocking Ro1 signaling with *nor*BIN. Ro1 mice taken off dox at weaning (P21) develop enlarged ventricles, further supporting the idea that hydrocephalus is a consequence of Ro1 expression.

At birth double tg mice off dox appeared grossly normal and were indistinguishable from their control littermates, but began to exhibit macrocephaly between P10 and P15. At P15 Ro1 mice have a lateral ventricle to brain ratio that exceeds 0.30 and by P30 have developed a severe hydrocephalus phenotype characterized by greatly dilated lateral and third ventricles that caudally displace surrounding brain structures. The rostral end of the Sylvius aqueduct is obliterated, with no lumen and no detectable ependymal cells visible in coronal sections of this

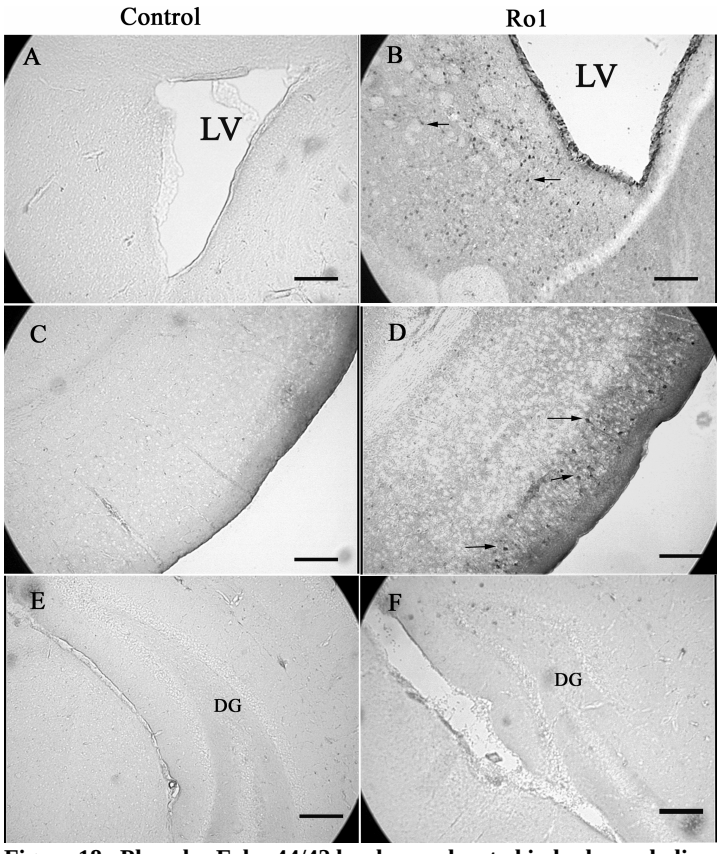

Figure 18. Phospho-Erk p44/42 levels are elevated in hydrocephalic Ro1 mice. Increased phospho-Erk p44/42 staining is observed around the lateral ventricles (***B***) and in the entorhinal cortex (***D***) of hydrocephalic Ro1 mice but not in littermate single-transgenic controls (P21) (***A,C***). No increases in phospho-Erk were observed in the hippocampus (***F***). Sections shown in ***A*** and ***B*** are at the level of the anterior commissure and striatum; ***C-F*** are at the level of the subcommissural organ. Scale bars, 150 µm. LV=lateral ventricle, DG=dentate gyrus.

region, suggesting a denudation of ependymal cells has occurred. The collicular recess and caudal end of the Sylvius aqueduct, however, remain open. The subcommissural organ (SCO) also appears disorganized in hydrocephalic mice, although it is unclear whether this is due to Ro1 expression or if it is a result of hydrocephalus.

It is unclear how, in the absence of applied ligand, Ro1 expression leads to the development of hydrocephalus. Ro1 is likely to be highly over-expressed relative to other astrocytic receptors. Since G-protein-coupled receptors have a low level of constitutive activity (Lefkowitz et al., 1993; Costa and Cotecchia, 2005), Ro1 may be expressed at a high enough level that its constitutive activity is sufficient to trigger a series of events that ultimately result in hydrocephalus. Ro1 may be also expressed at a high enough level that dynorphin or another ligand that normally would have a low affinity for Ro1 can bind and activate the receptor. Significantly, mice with Ro1 expression targeted to the heart developed cardiomyopathology when taken off dox. When these mice were given an intermediate dose of dox to reduce Ro1 expression, their heart rates normalized. Treating these mice with pertussis toxin or the KOR inverse agonist *nor*bin also restored normal heart rates, demonstrating that Ro1 signaling was responsible for the changes in heart (Redfern et al., 2000). A similar process where Ro1 overexpression and constitutive activity leads to pathology may be occurring in our mice.

Our finding is, to our knowledge, the first to implicate glial G_i signaling pathways in the development of hydrocephalus. Ro1 expression in astrocytes may contribute to the development of hydrocephalus by altering the extracellular matrix so that more fluid from the extracellular space enters the ventricles. It could also alter the levels of various neurotransmitters, growth factors, or cytokines in the CSF resulting in a disregulation of CSF

production by the choriod plexus. Astrocytic release of glutamine is known to be down regulated in the kaolin model of hydrocephalus (Kondziella et al., 2003); it is possible that increased G_i signaling in astrocytes may affect glutamine production or release that in turn changes neuronal input into the SCO. It is interesting to note that gene expression analysis in the H-Tx rat, which has primary stenosis of the aqueduct similar to Ro1 mice, has implicated several genes linked to astrocytes and G-protein coupled receptors, including connexin 30, beta integrin 5, and somatostatin (Jones et al., 2001b; Miller et al., 2006).

Although SCO secretory function could not be determined from our histological examination of Ro1 brains, the observation that the SCO is disorganized in Ro1 mice is significant. The presence of a normal SCO appears to be necessary for the development and maintenance of the aqueduct, as impaired SCO formation and/or function are found in multiple animal hydrocephalus models and in human infantile hydrocephalus (Overholser et al., 1954; Newberne, 1962; Takeuchi and Takeuchi, 1986; Jones et al., 1987; Jones and Bucknall, 1988; Perez-Figares et al., 1998; Louvi and Wassef, 2000; Estivill-Torrus et al., 2001; Sakakibara et al., 2002; Fernandez-Llebrez et al., 2004). The SCO secretes negatively charged glycoproteins, such as SCO-spondin and RF-Gly I, that appear to be critical for maintaining an open aqueduct by their physical presence. The SCO may also regulate CSF formation. Receptors for SCO glycoproteins are found on the choroid plexus (Miranda et al., 2001), suggesting that CSF production could be influenced by SCO activity.

We have shown that phosphoErk levels are increased in hydrocephalic mice. G_i-coupled receptors are known to signal via the MAP kinase pathway, thus Ro1 signaling may lead to changes in gene expression that contribute to the pathogenesis of hydrocephalus. We cannot rule out, however, the likely possibility that the observed increase in phosphoErk is a

consequence of hydrocephalus and not causal. Elevated phosphoErk has been associated with astrogliosis (Mandell and VandenBerg, 1999), a pathological response of astrocytes to many types of brain injury, including hydrocephalus (Fukumizu et al., 1996). Increased GFAP expression observed in severely hydrocephalic Ro1 mice is indicative of astrogliosis, particularly since GFAP levels are not elevated in Ro1 mice with mild hydrocephalus (data not shown).

The observed loss of ependymal cells in Ro1 mice is in agreement with other studies that demonstrate a denudation of ependymal cells in the ventricular system (Jimenez et al., 2001; Dominguez-Pinos et al., 2005). The underlying cause of ependymal detachment is unknown, although defects in certain adhesion molecules, such as L1, have been indicated in other HC models (Schmid et al., 2000; Itoh et al., 2004). As a subset of ependymal cells express GFAP (Takano et al., 1996; Rodriguez-Perez et al., 2003), it is possible that Ro1 may be expressed in these cells, causing a defect that leads to detachment. Ependymal cells may also be affected indirectly by changes in levels of secreted cytokines or altered neuronal inputs resulting from Ro1-induced changes in astrocyte signaling.

To develop more effective treatments, a better understanding of hydrocephalus pathogenesis is needed. The Ro1 model of hydrocephalus has several characteristics that will make it valuable for studying hydrocephalus. First, the Ro1 model does not require injection of foreign bodies into the ventricles to cause hydrocephalus, making the Ro1 model easier to work with and more relevant to human cases. Second, hydrocephalic animals are produced reliably; all Ro1 mice will develop hydrocephalus if maintained off dox. Inherited models of hydrocephalus, such as the H-Tx rat, reliably produce hydrocephalic offspring but at a much lower rate, and which animals will develop hydrocephalus cannot be predicted at early ages

(Jones et al., 2000; Jones et al., 2001b; Miller et al., 2006). Third, dox can be used to regulate when hydrocephalus will occur. It would be interesting to compare juvenile and adult onset to see if age changes how hydrocephalus develops. Fourth, the rate of development and the severity of hydrocephalus can be manipulated by giving Ro1 mice a lower concentration of dox. Slowing the development of hydrocephalus allows changes in the brain to be studied more thoroughly and could provide insights into how hydrocephalus progresses. The Ro1 model of hydrocephalus should prove useful in advancing our knowledge of hydrocephalus pathophysiology. Furthermore, the Ro1 model provides new evidence for the involvement of astrocytic G-protein coupled receptor signaling in the development of hydrocephalus.

CHAPTER 5
EPENDYMAL CELL GENOMIC ARRAYS

Although the cause of HC is multi-factorial, the progression of pathology appears remarkably similar across various models of the disease. A common signaling pathway(s) may underlie the pathology. If so, identification of the pathway(s) involved would allow new

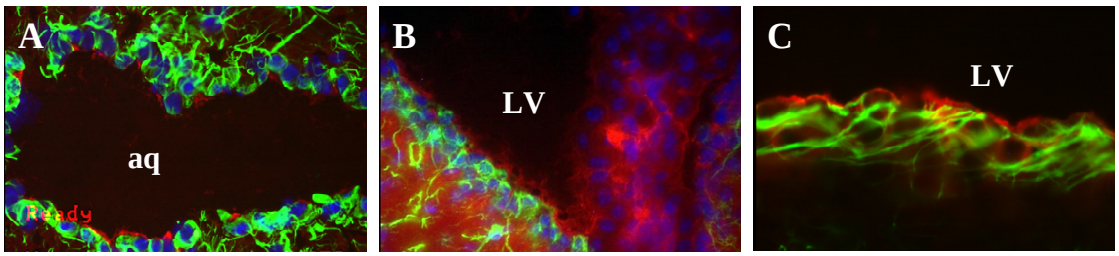

Figure 19. GFAP is expressed in ciliated ependymal cells. Coronal sections from control mice were stained with anti-GFAP (green) and α-tubulin (red) antibodies and counterstained with DAPI (blue). ***A*** Cells lining the aqueduct were positively labled with anti-GFAP antibodies. A subset of these cells was also labeled with the anti- α-tubulin antibody, a marker for cilia. ***B*** A subset of cells lining the lateral ventricles were also labeled with both anti-GFAP and anti- α-tubulin antibodies. GFAP-positive processes extending from the underlying subependymal layer to the lumen are also evident. The cells choroid plexus (right) express α-tubulin but not GFAP. ***C*** A higher magnification of anti-GFAP and anti- α-tubulin staining in the ependymal layer of the lateral ventricle. Aq=aqueduct, LV=lateral ventricle

pharmacological approaches for treating HC to be developed. Because onset of the disease in our model is controlled by dox, the Ro1 model provides the opportunity to look for the earliest changes in disease progression without the complications caused by later pathology.

Ependymal denudation is a common feature of HC that appears to happen early in the progression of the disease. Ependymal denudation is reported in most, if not all, HC models and human cases. Whether denudation occurs prior to ventricular dilation or as a result of dilation is still unsettled, however there is strong evidence that denudation occurs early in the disease process, and loss of the ependyma cell layer or cilia function is also sufficient to cause HC (Davy and Robinson 2003, Ibanez-Tallon et al 2002, Grondona et al 1996). In the

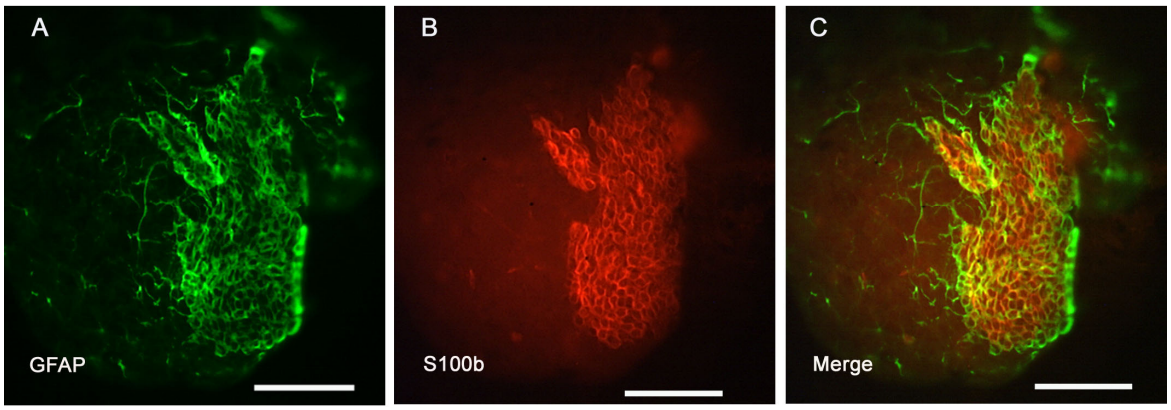

Figure 20. Isolated sheets of ependymal cells express GFAP and S100β. Sheets of the lateral ventricle ependymal cell layer were double-stained for GFAP (***A***) and S100β (***B***). An overlay of the staining shows most S100β positive cells are also GFAP positive (***C***). Scale bars: ***A,B,C*** 100 µM.

Ro1 HC model, expression of the G_i-coupled Ro1 receptor in GFAP positive cells results in HC, although how enhanced G_i signaling leads to HC is unknown. Ependymal denudation is also seen in the Ro1 model, prompting us to question whether Ro1 signaling cascades could lead to ependymal denudation and subsequent onset of HC. To address this question, we used Affymetrix gene arrays to look for changes in gene expression in ependyma shortly after turning on Ro1 expression.

Results

Expression of Ro1 in ependymal cells. Because of conflicting reports of GFAP expression in ependymal cells, we wanted to determine if ependymal cells express GFAP in our mouse line. Brains from adult mice were processed for immunostaining and cut into coronal sections. Sections containing lateral ventricles, third ventricle and aqueduct were probed with antibodies against GFAP and the ependymal cell marker S100β. A subpopulation of the ependymal cells was double-labeled with both markers, and the specialized ependyma of the SCO strongly expressed GFAP (Figure 19B). Additionally, in the lateral and third ventricles GFAP-positive processes could be seen wrapping around cell bodies in the subependymal cell layer and extending to the ventricular surface (Figure 19C). To further define the GFAP

labeled cells, sections were stained for GFAP and α-tubulin, which is highly expressed in cilia. The cells lining the ventricles and the cells of the choroid plexus were edged on the luminal side with positive α-tubulin staining. A subpopulation of the ependymal cells lining the lateral and third ventricles and the majority of ependyma lining the aqueduct were GFAP positive and labeled with anti- α-tubulin antibodies on the apical surface, suggesting that the GFAP expressing cells are ciliated ependymal cells. Cells of the choroid plexus did not express GFAP in any of the sections (Figure 19).

Secondary antibodies can sometimes bind nonspecifically to the edges of tissue, resulting in falsely labeled cells. To reduce the likelihood that edge effect could be influencing staining in sections, the ependymal cell layer was isolated in sheets from the lateral ventricles and placed on slides for staining. Most cells in the isolated sheets expressed S100β, confirming that they were ependymal cells, and the majority of S100β positive cells were also labeled with GFAP (Figure 20). α-tubulin staining of ependymal sheets revealed distinct tufts of cilia on the cells, and ciliated cells also expressed GFAP (Figure 21). Thus in the Ro1 mouse model at least a subpopulation of ependymal cells do express GFAP and would be able to express the Ro1 receptor.

To look for the Ro1 receptor expression in ependymal cells, double tg Ro1 mice and single tg littermate controls were taken off dox at P30 and sacrificed two to four weeks after dox removal. The Ro1 receptor is FLAG-tagged, so fresh

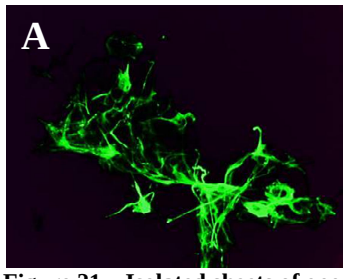

Figure 21 . Isolated sheets of ependymal cells express GFAP (green) **and α-tubulin** (red). ***B*** Tufts of cilia labeled with α-tubulin antibody clearly visible (arrow).

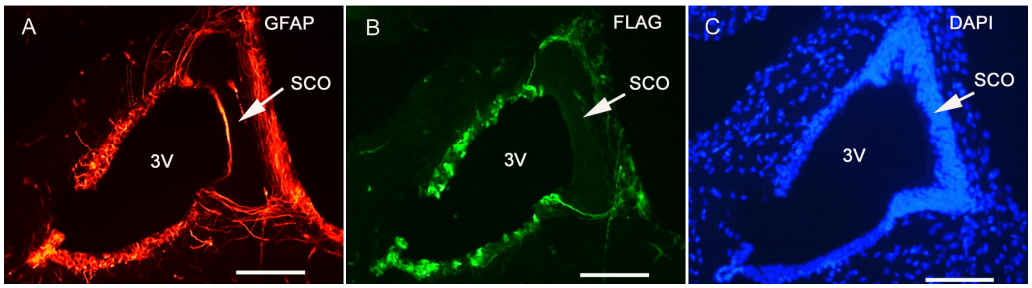

Figure 22. Ro1 is expressed in ependymal cells. Coronal brain sections of Ro1 mice off dox were double-stained for FLAG and GFAP and counterstained with the DNA stain DAPI. (***A***) Ependymal cells lining the third ventricle, but not cells within the SCO, express GFAP and FLAG (***B***). (***C***) DAPI staining shows the cells of the ependymal layer and SCO. 3V= Third ventricle, SCO=subcommissural organ. Scale bars: ***A,B,C*** 100µM

frozen coronal sections and isolated ependymal cell sheets were probed with anti-FLAG epitope and GFAP antibodies. A small percentage of ependymal cells expressing GFAP stained positively for FLAG in both brain sections (Figure 22) and ependymal sheets from double tg mice but not in any tissue from control mice, confirming that Ro1 is indeed

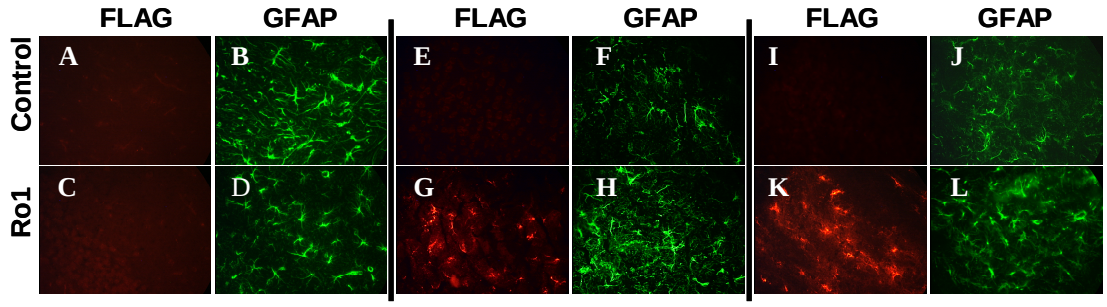

Figure 23. Time course of Ro1 expression after dox removal. ***A-D*** At day 0, no positive FLAG staining was seen in control (***A***) or Ro1 mice (***B***). ***E-H*** By day 3 a few cells in the thalamus of Ro1 mice are labeled with FLAG antibodies (***G***). ***I-L*** At 7 days off dox FLAG staining in the thalamus of Ro1 mice is increased and positively labeled cells are also found in the hippocampus and cortex (not shown). At each timepoint cells from control mice are negative for FLAG staining (***A,E,I***).

expressed in ependymal cells from double tg mice.

Timing of Ro1 expression and ependymal denudation. In order to look for early events in HC development, the timing of Ro1 expression following dox removal needed to be determined. Double tg Ro1 mice and litter mate controls were taken off dox at P30 and

brains collected at day 0, day 3, and day 7 after dox removal. Fresh frozen coronal brain sections were probed for the FLAG epitope (Figure 23). On day 0, no FLAG staining was observed in either double or single tg mice. By day 3, positive FLAG staining was visible mainly in the thalamus of double tg mice, and by day 7 positive FLAG staining was found in the thalamus, hippocampus, and cortex. At no timepoint did FLAG antibodies positively label cells from single tg mice.

Onset of ependymal denudation after dox removal was determined by H&E staining of coronal brain sections. Double tg Ro1 mice and littermate controls were taken off dox at P30 and pairs were killed at day 3, day 6, day 10, and day 14. Ten days after dox removal small patches of ependyma lining the septal wall of the lateral ventricles were missing or detached from the underlying parenchyma in Ro1 mice. Most of the ependymal cells remained attached and retained a cuboidal shape, and there was no evidence for ependymal detachment in the aqueduct. Single tg mice did not have ependymal detachment at any timepoint. Thus ependymal detachment begins by 10 days after dox removal in double tg Ro1 mice.

Affymetrix gene arrays. Genomic arrays were utilized to identify target ependymal genes that may be responsible for subsequent cell detachment. To avoid gene changes that result as a consequence of HC, ependymal

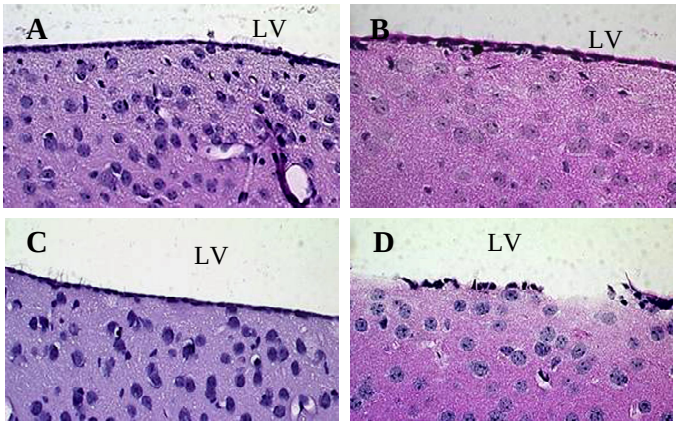

Figure 24. Time course of ependymal denudation after dox removal. Coronal sections from control (***A,B***) and Ro1 mice (***C,D***) were stained with hemotoxylin and eosin to look for ependymal denudation at 6 days (***A,C***) and 10 days (***B,D***) after dox removal. At six days off dox ependyma are normal in both control (***A***) and Ro1 mice (***C***). Patches of ependymal denudation are evident at 10 days off dox in Ro1 mice (***D***) but not in littermate controls (***B***).

cells needed to be collected very early in the disease process, but not so early that changes have not yet occurred. Based on the time course of Ro1 expression and ependymal denudation, day 5 and day 9 after dox removal were chosen as the timepoints to be used in gene arrays. Expression of Ro1 in double tg ependyma at these timepoints was confirmed using RT-PCR. Double tg (n=3) and single tg (n=3) mice were taken off dox at P30 and sacrificed at P35; four double tg and four single tg mice were taken off dox at P30 and sacrificed at P39. Approximately 0.5-1.0 µg of total RNA was extracted from the isolated ependymal cells of each animal and amplified for use in Affymetrix Mouse GeneChip Gene 1.0ST arrays. All chips passed internal quality controls, and the data was sent to Expression Analysis, Inc. for statistical analysis. Expression data for each genotype and timepoint was averaged together and two-group comparison with permutation analysis for differential expression (PADE) was used to identify differentially expressed genes. Using the criteria of p-value $\leq$ 0.05 and fold change $\geq$ 0.20, 97 genes were differentially expressed at 5 days off dox and 343 genes were differentially expressed at 9 days off dox. Most gene changes were small. At 5 days off, the largest fold change was .67 and at 9 days off dox only 37 genes where changed by more than 20%. However, no genes were found to be differentially expressed with a false discovery rate (FDR) less than one, meaning that all changed genes are likely not truly different between double and single transgenics.

RT-PCR validation. Twelve genes from each timepoint were selected based on p-value and biological relevance for RT-PCR validation (Table 2). There was no significant difference between Ro1 and control mice for any of the selected genes, even when ependymal cells from multiple mice (same genotype) were combined to generate sufficient total RNA for

efficient reverse transcription. We were therefore unable to interpret the gene array data or draw any conclusions about the effect of Ro1 signaling in ependymal cells.

Discussion

Similar to other HC models, ependymal denudation is a characteristic of HC in our Ro1 mouse model of HC. Because ependymal denudation is sufficient to cause HC (Grondona et al., 1996), we wanted to use gene arrays on ependymal cells to see if overexpression of Ro1 leads to changes in gene expression that ultimately causes denudation. Although some groups have not been able to detect GFAP in ependyma, other groups have shown that a subset of ependyma do express GFAP. The conflicting reports have been suggested to result from differences in fixation and staining methods, age, and type of animal (Bruni et al., 1985; Kasper et al., 1991; Sarnat, 1992; Xiuxin Liu, 2006). Immunostaining of adult ependyma in tissue sections and isolated ependymal sheets from our mice showed that a percentage of ciliated cells expressing the ependymal cell marker S100β also expressed GFAP. Thus in the Ro1 mice, we also detected GFAP in a subpopulation of ependymal cells similar to previous reports. More detailed studies would need to be carried out to determine the percentage, although GFAP expression seems to be higher in the SCO and aqueduct. Since ependymal cells do express GFAP, Ro1 should be expressed in the ependyma of double tg mice. Immunostaining for the FLAP epitope confirmed that at least a small subset of ependymal cells express the Ro1 receptor. This finding is not unexpected since not all ependyma detach during HC. If Ro1 expression does cause denudation in our model, then it is possible that GFAP could be a marker for the subsets of cells that detach in HC in other models.

Selecting timepoints for gene arrays was of some concern. The Ro1 model allows the disease process to be started by removing dox and turning on Ro1 expression, but the time required for dox to be cleared from the CNS and Ro1 expression to begin was unknown. If too early a timepoint was selected, Ro1 would not be expressed or would not have been expressed long enough for changes in gene expression to occur. If too late a timepoint was chosen, the early events of HC would be missed, and ependyma may have started to detach, making tissue collection extremely difficult. Timed studies of Ro1 expression and ependymal denudation after dox removal in double tg mice showed that Ro1 is expressed in the thalamus as early as three days after dox removal. Although isolated ependymal sheets were not included in the timed staining studies, subsequent RT-PCR of double tg ependyma at 5 and 9 days off dox showed that Ro1 can be detected at both timepoints and is more highly expressed at the 9 day timepoint, suggesting that dox is still being eliminated from the brain. H&E staining from similar timed studies showed normal ependyma in double tg mice at 3 and 6 days off dox. At ten days off dox, small areas of detaching ependyma were seen in the lateral ventricle. However, it should be noted that only two mice were examined in this study, and disease progression is highly variable between animals. Based on the staining results, 5 and 9 days off dox were selected for the genomic array timepoints.

5 Days Off Dox	9 Days Off Dox
TNFα	MID1
Caspase 8	MNS1
SIX2	CABLES2
SLC17A6	SCP2
TRHR	DSC3
LRP2	LRP2
MYL1	GFRA2
ITGAX	HYDIN
C1S	EPS8L1
ST8S1A6	ADAMTS3
PLEC1	TNFAIP6
	CSF3

Table 2. Gene targets for RT-PCR validation

The gene array data cannot be interpreted since no differentially expressed genes between double tg mice and single tg controls at either timepoint could be validated by PADE analysis (for false discovery) or by RT-PCR. Although not many changes were expected to be found, the lack of differences is surprising and could be attributed to several different factors. First, Ro1 expression in ependyma may not cause changes at the gene level at these timepoints. Instead, Ro1 signaling may alter other signaling pathways that respond rapidly and result in denudation. Ro1 signaling in ependyma may not be the causal factor in detachment. Rather, Ro1 signaling in other cell types could lead to detachment. It is also possible that although Ro1 expression was detected in the brain at three days off dox, ependymal cells would be exposed to dox longer through contact with the CSF and thus not begin expressing Ro1 at high enough levels to cause pathology until later timepoints. Alternatively, the percentage of cells expressing Ro1 in the ependymal isolations may be small enough that changes in gene expression actually occurring could not be detected. The large variability in rate of disease progression between animals could also make actual changes in gene levels hard to detect statistically without using a much larger sample size. The lack of changes could also be due to technical issues. The amount of total RNA submitted in each sample was very small, less than 1 µg. The necessary amplification steps could skew the relative levels of transcripts used in the array. Although data from the arrays cannot be interpreted, it is intriguing that a number of genes differentially expressed (by student's t test) were genes related to cilia function, extracellular matrix, and cell adhesion. However, these data cannot be interpreted, and further work is required to determine what changes, if any, Ro1 signaling causes in ependymal cells.

CHAPTER 6
DISSCUSSION

Despite decades of neuroscience research, many neurological disorders remain poorly understood. Interest in the role of glia in both normal and pathological states is increasing, particularly as evidence is presented for astrocyte modulation of neuronal activity. Recent research has provided evidence that astrocytes may have a greater function beyond providing structural and metabolic support for neurons. First, astrocytes account for approximately half of the brain's volume and are found in every part of the CNS. Many neuronal processes and synapses are surrounded by astrocytes; a single astrocyte can envelop as many as 80,000 synapses (Bushong et al., 2002). Second, astrocytes and neurons express a similar complement of receptors for neuroligands (Porter and McCarthy, 1996) and astrocytes are able to respond to numerous stimuli with an increase in intracellular calcium (Araque et al., 2001), which is generally accepted to be a form of glial excitability (Hansson and Ronnback, 2003). Third, astrocytes also appear to release gliotransmitters, including glutamate and ATP. For example, a calcium increase in astrocytes evokes an inward current in adjacent neurons that is dependent on glutamate release (Parpura et al., 1994; Carmignoto et al., 1997; Araque et al., 1998; Pasti et al., 2001) and uncaging IP_3 in a single astrocyte causes a glutamate-dependent increase in AMPA mEPSC frequency in a neighboring neuron (Fiacco and McCarthy, 2004). Yet little is actually known about what role astrocytes may play in listening to and modulating neuronal communication under physiological conditions.

Because an astrocyte-specific receptor and ligand have yet to be discovered, astrocytic responses cannot be isolated pharmacologically. The Ro1 transgenic mouse line was created to manipulate signaling events specifically in astrocytes in order to investigate the role of astrocyte GPCR signaling in modulating behavior and neuronal activity.

Targeting a G_i-coupled receptor to astrocytes. The tetracycline transactivator (tTA) system was used to conditionally express Ro1 in astrocytes on a kappa opioid receptor knockout background. Ro1 is a kappa opioid receptor modified by replacing the second extracellular loop with the second extracellular loop of the delta opioid receptor. Ro1 has a greatly reduced affinity for endogenous ligands but is still activated by the highly selective small molecule drug spiradoline (Coward et al., 1998; Redfern et al., 1999). To restrict Ro1 expression to astrocytes, transgenic (tg) mice expressing the tetracycline transactivator (tTA) under the control of hGFAP, an astrocyte-specific tissue promoter, were crossed with a second line of mice carrying the Ro1 and LacZ genes under the control of the tet operon (tetO) promoter. Timing and level of Ro1 expression can be controlled with doxycycline, thus any potential developmental side effects of Ro1 expression could be eliminated by maintaining mice on doxycycline until maturity. Using spiradoline to activate Ro1 would allow the study of astrocytic G_i-coupled signaling on animal behavior and neuronal activity.

Ro1 expression and cellular localization. Immunoprecipitation assays and βgal staining demonstrated that Ro1 is expressed only in double tg mice (positive for both tTA and Ro1) and only when the animals are maintained in the absence of dox, confirming that Ro1 expression is indeed regulated by doxycycline without any apparent “leakiness”. Cellular localization of Ro1 was determined using immunocytochemistry; the localization of Ro1

immunostaining to GFAP positive cells and its absence in NeuN positive cells confirmed that Ro1 expression is being driven by the GFAP promoter. Because spiradoline, the synthetic ligand for Ro1, activates the kappa opioid receptor, the Ro1 genotype was moved onto a KOR knockout background to ensure that the only response to spiradoline is mediated by Ro1.

G_i signaling and G_i modulation of behavior. G protein coupled receptors (GPCRs) are a class of seven transmembrane spanning receptors that are important mediators of intracellular signaling. The cytoplasmic tail of a GPCR interacts with the heterotrimeric G proteins, which are composed of α, β and γ subunits. In an inactive state, the α subunit binds GDP. Activation of the GPCR causes a conformational change in Gα, allowing GDP to be replaced with GTP and releasing the Gβγ dimer. There are multiple families of Gα that couple to different effector molecules. $G\alpha_i$ is so named for its inhibition of adenylate cyclase and subsequent reduction of cAMP formation. A number of studies suggest that G_i-coupled receptors in the CNS, including dopamine, serotonin, opioid and muscarinic acetylcholine receptors have roles in regulating both behavior and neuronal activity in normal and pathological states. The focus of many of these studies has been on neuronal receptors even though astrocytes also express many of the same receptors and there is indirect evidence that activation of astrocytic receptors may play a role in the observed effects. The Ro1 mice were designed to allow us to directly test if G_i signaling in astrocytes alone can modulate behavior or neuronal activity.

Alterations in G_i protein levels and activity have been implicated in multiple pathological states, including depression and panic disorders (Joseph et al., 1993; Saitoh et al., 1993; Gurguis et al., 1999c; Gurguis et al., 1999e; Gurguis et al., 1999d; Gurguis et al.,

1999b; Gurguis et al., 1999a). There is a significant reduction in stimulated cAMP formation in frontal cortex tissue from human suicide victims (Valdizan et al., 2003) and both increases (Garcia-Sevilla et al., 1999) and decreases (Pacheco et al., 1996) in G_i protein levels have been found in the brains of depressed subjects. As these studies were done on brain tissue lysates, neuronal and astrocytic components cannot be determined. Dopamine D2 receptors signal primarily via G_i pathways. Neuroleptic antipsychotic drugs, which are primarily dopamine D2 receptor antagonists, are used to treat depression and panic disorders. Since nearly 1/3 of all D2 receptors in the cerebral cortex are located on astrocytes and astrocytic processes rich in D2 receptors surround cortical interneurons lacking D2 receptors (Khan et al., 2001), astrocytes seem to be ideally placed to mediate some of the therapeutic effects. Furthermore, the D2 agonist quinpirole causes an increase in intracellular calcium in cultured cortical astrocytes that can be blocked with the D2 antagonist raclopride (Khan et al., 2001); increases in astrocytic calcium are correlated with changes in neuronal activity and release of gliotransmitters (Pasti et al., 1997; Newman and Zahs, 1998; Parri et al., 2001). It is possible that activity at astrocytic dopamine receptors accounts for at least part of the effect of anti-depressant and antipsychotic drugs, and therefore defects in astrocytic G_i signaling could have a critical role in these disorders.

G_i modulation of neuronal excitability. G_i-receptor signaling is also associated with changes in neuronal activity. For example, kappa opioid receptor activation *in situ* inhibits glutamate synaptic currents presynaptically (Svingos and Colago, 2002; Bie and Pan, 2003), reduces NMDA mediated glutamatergic transmission (Svingos and Colago, 2002), prevents glutamate release evoked by potassium (Nicol et al., 1996), and inhibits NMDA mediated neurotransmitter release (DA and Ach) (Schoffelmeer et al., 1997). Dopamine receptor

agonists also suppress NMDA neurotransmission (Wang et al., 2006). While there is little question that neuronal receptors are involved in the observed results, astrocytes also express kappa opioid (Bunn et al., 1985; Eriksson et al., 1990; Belcheva et al., 2005) and dopamine receptors (Miyazaki et al., 2004), and activity at astrocytic G_i receptors could be involved in modulating the observed neuronal responses. As astrocytes express a number of different G_i- and G_s-coupled receptors it is likely that G_i signaling is important in regulating processes that effect astrocyte-neuron communication. For example, astrocytes respond to neuronal vasoactive intestinal peptide (VIP), a G_s-coupled receptor, with a release of neurotrophic factors, including activity-dependent neurotrophic factor (ADNF) (Gressens et al., 1997). ADNF causes the secretion of neurotrophin 3 in neurons and may function in long term potentiation by modulation of NMDA subunits 2A and 2B (Blondel et al., 2000). Since VIP receptors in astrocytes are G_s-linked GPCRs, activation of G_i could inhibit the release of ADNF and thus suppress long term potentiation induction.

Mice expressing Ro1 develop hydrocephalus. The original goal in creating the Ro1 mice was to allow selective activation of G_i signaling in a population of astrocytes while recording any subsequent changes in neuronal excitability or performing behavioral tests. However, severe hydrocephalus was observed in 100% of double tg mice off dox (expressing Ro1). All double tg mice maintained off dox developed visibly enlarged heads and had a 50% survival rate at 12 weeks of age, while all double tg mice on 25 µg/mL dox were normal with a 100% survival rate at 12 weeks, demonstrating that suppression of Ro1 expression prevents hydrocephalus. Control mice heterozygous for the Ro1 gene but lacking tTA (GFAP-tTA$^{-/-}$ -

tetO:Ro1$^{+/-}$) did not develop hydrocephalus, making it unlikely that the Ro1 gene is inserted in some critical location that results in hydrocephalus independent of receptor expression.

Doxycycline dosing. The appearance of hydrocephalus in double tg mice posed a serious problem for future studies of Ro1 signaling in astrocytes as differentiating between physiological or pathological responses would be impossible. The Conklin group had similar problems with G_i associated pathology when they over-expressed Ro1 in heart tissue, but were able to eliminate pathology in the heart by administering an intermediate dose of doxycycline to their animals (Redfern et al., 2000). Extensive attempts were made to determine a minimum dose of doxycyline that would eliminate hydrocephalus while still allowing expression of Ro1 at a detectable level. Double tg mice maintained on 1, 2, 5, 10, and 12 μg/mL doxycyline developed noticeably enlarged heads and had enlarged lateral ventricles, although with increasing age of onset (unpublished observations). All double tg mice maintained on 17 μg/mL dox appeared outwardly normal but had enlarged lateral ventricles at p60, yet double tg mice on 25 μg/mL dox had normal ventricles at p60. The relatively small change in dox concentration between healthy and affected double tg mice, coupled with difficulty in detecting the Ro1 receptor in mice maintained on 17 μg/mL dox, prompted us to focus on the Ro1 mouse line as a HC model.

The Ro1 model. The unexpected development of HC in the Ro1 mouse line has resulted in a unique and valuable model of HC. The pathology of HC in the Ro1 model is very similar to other HC models with stenosis of the aqueduct. HC appears to develop postnatally in our model as newborn double tg pups (maintained off dox) are indistinguishable from control

littermates. At birth the ventricles are not enlarged, the ependymal cell layer appears intact and brain structures appear grossly normal. After birth HC develops progressively; by p15 double tg pups have domed skulls and significant enlargement of the lateral and third ventricles, optic chiasm and pinal recess. Similar to other HC models, discontinuous loss of ependymal cells is seen throughout the lateral ventricles, third ventricle and the aqueduct. Damage to periventricular white matter is prominent with thinning of the cortex and posterior displacement of the hippocampus. The cerebellum is smaller but appears otherwise normal, the SCO is small and disorganized, and the aqueduct is obliterated (Sweger et al., 2007). Many of these pathological changes are common in HC, both in experimental models and in human cases.

Perhaps it is not surprising that pathology resulting from accumulation of CSF would be similar across models. However, the initiating events in HC are still unclear, and the underlying mechanisms that cause neurological and motor deficits in patients are poorly understood. The similarities between models suggest that there may be a common pathway or process that causes HC when disrupted. Identification of such a process could provide the opportunity to develop sorely needed pharmacological treatments for HC. A number of other HC models already exist, but all have limitations that make isolating initiating events in HC difficult. In spontaneous models, onset of HC cannot be controlled and penetrance is not 100%. Thus the animals that will develop HC cannot be predicted, limiting studies to the pathology and not the causes of HC. In induced models, the onset of HC is controlled but does not reflect congenital HC and may be complicated by inflammatory responses. Other transgenic mice develop HC as part of their phenotype, but the presence of other pathologies or developmental abnormalities makes identifying the processes responsible for HC difficult.

In the Ro1 model HC is initiated by disruption of G_i signaling, HC is the only pathology, onset of pathology is controlled, the rate of HC progression can be manipulated, and both congenital and adult onset HC can be studied. Thus the Ro1 model presents a new tool for understanding the molecular mechanisms of HC.

Ro1 signaling. Although the rate of HC development varies between animals, all double tg mice off dox will develop severe HC despite the absence of the Ro1 agonist spiradoline. GPCRs have basal levels of intrinsic signaling that are not blocked by an antagonist but can be blocked with an inverse agonist (Leurs et al., 2000; Kenakin, 2004; Wang et al., 2007). Because Ro1 is based on the kappa opioid receptor (KOR), a GPCR, basal signaling through Ro1 is the most likely explanation for development of HC in our model. *Nor*Binaltorphimine (*nor*BNI) is a KOR inverse agonist frequently used in opioid studies (Messer, 2004; Wang et al., 2007) and has been shown to be highly selective for KOR over other opioid receptors. A single dose can inhibit KOR signaling for weeks (Horan et al., 1992; Butelman et al., 1993; Powell and Holtzman, 1999; Ko et al., 2003; Messer, 2004), making *nor*BNI an ideal drug for long-term studies. Ro1 retains the binding site for *nor*BNI, and double tg mice treated with *nor*BNI after dox removal failed to develop ventriculomegaly or ependymal denudation after 30 days. Thus the signaling cascades activated by Ro1 are required for HC to occur; expression alone of Ro1 is insufficient to cause HC.

Elucidating the signaling pathways responsible for HC could provide valuable insights into HC pathology. While we know that Ro1 signaling triggers HC, it is unclear which cell types are primarily affected and should be the focus of further studies. Although GFAP positive cells are mostly astrocytes, astrocytes are not a homogenous population and

carry out a variety of diverse functions throughout the entire CNS. Other cells, including progenitors, ependymal cells and microglia, can express GFAP, particularly while undergoing development and maturation. A number of important postnatal developmental processes such as maturation of ependymal cells and cilia development occur during the first two postnatal weeks, coinciding with onset of HC in our model. However, it is unlikely that disruption of developmental processes by Ro1 signaling is the primary cause of HC because adult double tg mice kept on dox until p30 will also develop significantly enlarged ventricles and stenosis of the aqueduct when dox is removed. The rate of HC development is slower and variations in severity are more pronounced in adult onset, possibly reflecting differences in either genetic susceptibility or ability to compensate, yet the fact that even in adult onset all double tg mice develop HC indicates that Ro1 signaling is disrupting a critical process in normal CNS function.

Possible mechanisms. Ependymal cells are one of the cell types possibly affected by Ro1 expression. Ependymal denudation is seen across multiple experimental models of HC and in human cases. In at least one mouse model with stenosis of the aqueduct, denudation occurs in a distinct pattern early in the disease process, suggesting that denudation is not the result of stretching and could be the cause of HC (Raimondi et al., 1976; Jimenez et al., 2001). Other studies using neuraminidase to pharmacologically detach ependyma resulted in HC (Grondona et al., 1996). Because denudation was seen in Ro1 HC mice regardless of severity, we decided to focus on ependymal cells. While there is some contradiction in the literature regarding GFAP expression in ependyma (Bruni et al., 1985; Kasper et al., 1991; Sarnat, 1992; Xiuxin Liu, 2006), we were able to detect GFAP in ependymal cells by

immunocytochemistry. GFAP was not expressed by all ependyma, but ependyma are known to be a heterogeneous cell population, a fact that seems to be reflected in the discontinuous denudation seen in HC. Subsequently Ro1 expression in ependyma was confirmed by both immunocytochemistry and RT-PCR.

The consequences of Ro1 signaling in ependyma are not known. The classical action of $G\alpha_i$ is to inhibit $G\alpha_s$-stimulated production of cAMP by adenylyl cyclase. While the functional consequences of altered cAMP levels are difficult to predict, both gain and loss of the G_s-coupled PAC1 receptor results in dysfunctional cilia, and abnormal cAMP and phosphoCREB levels in ependyma are found in some HC models (Otto et al., 2004; Banizs et al., 2005; Lang et al., 2006). Long term $G\alpha_i$ activation also can sensitize some adenylyl cyclase isoforms, again with unknown functional effects (Thomas and Hoffman, 1996). More recently it has become clear that GPCRs have signaling functions outside of classical signaling pathways. $G\alpha_i$ activates Rho via Rho guanine nucleotide exchange factors (Billington and Penn, 2003) and mitogen-activated protein kinase (MAPK) pathways can be activated via cross-talk of GCPRs with receptor tyrosine kinases and focal adhesion complexes, leading to transcriptional activation. Thus unregulated Ro1 signaling could result in abnormal gene transcription in ependyma, possibly resulting in denudation. GPCRs can act independently of G proteins, participating in protein signaling complexes with beta arrestins. These signaling complexes also activate MAPK pathways, but with a slower and longer-lasting activation that is concentrated mainly in the cytosol (Luttrell, 2005). The ERK1/2 (extracellular signal-regulated kinase) MAPK pathway regulates a number of processes, including proliferation and migration. Detachment could result from changes in adhesion molecules or gap junction proteins; other structural proteins could be affected,

including actin, myosin, tau proteins or microtubules. Changes in gene expression or signaling pathways that impact cilia function could also lead to HC in the Ro1 model.

Gene arrays. The decision was made to use gene arrays to determine what, if any, changes in ependymal gene expression are caused by Ro1 signaling. While other groups have done gene arrays on tissue collected from various brain regions from HC animals (Balasubramaniam and Del Bigio, 2002; Morgan et al., 2005; Miller et al., 2006; Ballarati et al., 2007; Nonaka et al., 2008; Zhang et al., 2008), our goal was to look for changes occurring in a specific cell type before the onset of HC. Thus the ependymal cells needed to be collected soon after onset of Ro1 expression to avoid changes that may occur in response to other pathology. The time course for dox to "wash" out of the CNS was unknown, so timed studies were done to look for Ro1 expression and ependymal detachment following removal of dox in adult mice. The appearance of Ro1 after just three days off dox was surprising as we had anticipated that the washout time would be longer. The mice had always been on dox, and dox is known to accumulate in bone. However, Ro1 expression was limited to the thalamus at 3 days off dox and no morphological changes of the ependyma could be detected. By seven days off dox Ro1 was seen in other areas of the brain, including the hippocampus and cortex, and detachment of ependyma was first seen in histological studies at 10 days off dox. Based on these results, 5 and 9 days after dox removal were the time points selected for gene arrays.

Unfortunately, the array data proved to be unusable with no genes being statistically differentially expressed between double tg mice and littermate controls at either time point. We do not interpret the data to mean that no changes occur in ependymal cells, but that the

design of the experiment was flawed and therefore no conclusions can be drawn from the data. Although no significantly changed genes were found to have a false discovery rate of less than one, pathway analysis (using the genes that were significantly changed between genotypes) found significant enrichment of pathways potentially relevant for denudation, including pathways for adhesion, cilia function, matrix proteins, and integrin signaling, suggesting that real changes may be occurring but cannot be adequately detected in our samples. This may be due to improper time point selection. The timed studies were done on a very limited number of animals and did not extend longer than 7 days for immunostaining or 9 days for RT-PCR. It is very likely that the range of animal to animal variability of Ro1 expression was not captured in these studies, and thus biological variation between our samples prevented the statistical detection of actual changes. Higher levels of Ro1 RNA were detected with RT-PCR at 9 days off dox than at 5 days off dox, suggesting longer time courses using RT-PCR would be very useful in determining the maximal level of Ro1 expression in ependymal cells so we would better understand if the levels seen at 5 and 9 days off dox should be sufficient to detect changes if present. Another source of error could come from the cellular composition of our samples. Isolation of the ependymal cell layer is technically challenging, and inclusion of some underlying subependymal cells is unavoidable. If actual changes in ependymal cells at these timepoints are small, the presence of another cell type could potentially dilute the sample.

Several attempts were made to verify by RT-PCR changes in selected genes from each gene array. The initial experiments proved to be unreliable due to inefficient reverse transcriptase activity resulting from very low amounts of total RNA used. Subsequent attempts using total RNA combined from three mice per sample produced more reliable

results, but no significant changes were found in the expression of the selected targets. Using a higher n would perhaps balance out biological variability, however, based on the questionable array data and preliminary RT-PCR results continuing the present study did not seem prudent.

Alternate hypotheses. Ro1 signaling may not cause changes at the transcriptional level in ependymal cells; denudation may instead result from altered signaling pathways in the cytosol. It is also possible that ependymal cell denudation is not the cause of HC in the Ro1 model. Rol signaling could impact the function of the subcommissural organ (SCO). Cells of the SCO strongly express GFAP, and Ro1 signaling in these cells could alter their secretory activity. The glycoproteins secreted by the SCO are theorized to play critical roles in keeping the narrow aqueduct patent and possibly in regulation CSF formation by the choroid plexus, thus loss of glycoprotein secretion could result in stenosis of the aqueduct and onset of HC. Ro1 signaling could also change the ability of astrocytes to carry out normal functions in the CNS. Astrocytes are found throughout the parenchyma and also directly contact the endothelial cells of blood vessels. Given the emerging importance of CSF absorption into venous blood, changes in astocytic G_i signaling that impact the ability of CSF to move through the interstitial spaces or to be absorbed into blood vessels, perhaps by increasing production of extracellular matrix proteins (as seen in the S100β tg model) or by causing constriction of blood vessels, could result in the accumulation of CSF and onset of HC.

Future directions. The Ro1 HC model presents the field an opportunity to study the earliest events in HC pathology. Although treating HC by shunting has greatly improved the life expectancy and quality of life for patients with HC, new treatments capable of preventing neurological damage or motor deficits are greatly needed. Future studies of the Ro1 model should focus on understanding the Ro1 receptor and how enhanced G_i signaling in GFAP positive cells results in HC. While the affinity of Ro1 for endogenous KOR ligands is significantly reduced, overexpression of Ro1 may allow activation of the receptor by ligands that normally would not bind. Using a KOR antagonist, other than norBIN (an inverse agonist), to see if blocking signaling while maintaining intrinsic activity of Ro1 still results in HC would provide insight into whether Ro1 may be activated by endogenous ligands in our model.

Further work is needed to determine if ependymal cells are primarily involved in HC pathology. The timed studies previously mentioned should be expanded to gain a better understanding of the time range needed for Ro1 expression and ependymal denudation to begin after dox removal. Included in these studies should be immunostaining time courses designed to look for any set patterns of Ro1 expression, as well as RT-PCR experiments for detecting Ro1 levels in ependyma. Ependymal cells should be collected at the new time points established from these experiments and studied for changes at the kinase/phosphatase or protein levels.

To determine if SCO functions are impacted by Ro1 signaling, anti-RF antibodies could be used to look for changes in SCO secretions. If differences in glycoproteins are seen in Western blots of CSF from sick animals, a timed study should be done to see when these changes occur in relation to pathology. Changes in protein levels or phosphorylation of

proteins involved in vesicle docking and fusion could also be examined to see if secretory functions are impaired in Ro1 mice. PNA and RCA lectin binding to ependymal surfaces could also be used to determine if glycosylation is normal in ependyma.

Other potential areas of study include the progenitor cells of the subventricular zone (SVZ), cilia function, and extracellular matrix formation around blood vessels. Progenitor cells in the SVZ, specifically the B cells, express GFAP and thus should also express Ro1. Changes in the proliferation of progenitors may contribute to the pathology of HC; BrdU incorporation experiments in both immature and adult mice could provide insight on the impact of G_i signaling in these populations. Immunostaining for SVZ cell markers could show alterations in relative numbers of A, B, and C cells and potential changes in migration. Previous α-tubulin and H&E staining of double tg mice showed that cilia are present on ependymal cells, but did not provide information on whether cilia structure is normal. Using brain slices and fluorescent dyes in a fluid chamber would show if cilia are able to beat normally. Additional immunostaining using antibodies against various cilia proteins would also help identify any abnormalities in cilia structure and function. Changes in extracellular matrix proteins could also be detected by Western blots and immunostaining.

The development of hydrocephalus in the Ro1 transgenic mouse has resulted in a new inducible model for hydrocephalus with the potential to provide new insights into the causes of this disease. Finding pharmacological treatments for HC has been hindered by a lack of understanding of the molecular pathways that cause HC and are responsible for the neurological and motor deficits that frequently accompany the disease even after successful shunting. We have characterized the Ro1 model, confirming that the GFAP promoter drives expression of Ro1 and determining that Ro1 signaling is causing the onset of HC. While

some of the initial attempts at elucidating the signaling pathways perturbed by Ro1 were unsuccessful, the Ro1 model remains an exciting tool for HC research and future studies with the Ro1 mice have the potential to impact the course of HC treatment.

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